

09/29/00

JCS 803 U.S. PAT

**UTILITY
PATENT APPLICATION
TRANSMITTAL**

TRANSMITTAL
(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No. 195378US0DIV

First Inventor or Application Identifier Chieko OSUMI

Title RAFFINOSE SYNTHASE GENE, METHOD OF PRODUCING RAFFINOSE, AND TRANSGENIC PLANT

JC675 U.S. PRO
09/09/09 09/29/00

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Docket No. 195378US0DIV

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

INVENTOR(S) Chieko OSUMI et al.

SERIAL NO: New Application

FILING DATE: Herewith

FOR: RAFFINOSE SYNTHASE GENE, METHOD OF PRODUCING RAFFINOSE, AND TRANSGENIC PLANT

FEES TRANSMITTAL

ASSISTANT COMMISSIONER FOR PATENTS
WASHINGTON, D.C. 20231

FOR	NUMBER FILED	NUMBER EXTRA	RATE	CALCULATIONS
TOTAL CLAIMS	24 - 20 =	4	× \$18 =	\$72.00
INDEPENDENT CLAIMS	7 - 3 =	4	× \$78 =	\$312.00
<input type="checkbox"/> MULTIPLE DEPENDENT CLAIMS (If applicable)			+ \$260 =	\$0.00
<input type="checkbox"/> LATE FILING OF DECLARATION			+ \$130 =	\$0.00
			BASIC FEE	\$690.00
			TOTAL OF ABOVE CALCULATIONS	\$1,074.00
<input type="checkbox"/> REDUCTION BY 50% FOR FILING BY SMALL ENTITY				\$0.00
<input type="checkbox"/> FILING IN NON-ENGLISH LANGUAGE			+ \$130 =	\$0.00
<input type="checkbox"/> RECORDATION OF ASSIGNMENT			+ \$40 =	\$0.00
			TOTAL	\$1,074.00

- Please charge Deposit Account No. 15-0030 in the amount of A duplicate copy of this sheet is enclosed.
- A check in the amount of **\$1,074.00** to cover the filing fee is enclosed.
- The Commissioner is hereby authorized to charge any additional fees which may be required for the papers being filed herewith and for which no check is enclosed herewith, or credit any overpayment to Deposit Account No. 15-0030.
A duplicate copy of this sheet is enclosed.

Respectfully Submitted,

OBLON, SPIVAK, McCLELLAND,
MAIER & NEUSTADT, P.C.

Date: 9/28/00



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Docket No. 195378US0DIV

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF :

CHIEKO OSUMI ET AL : ATTN: APPLICATION DIVISION

SERIAL NO: NEW APPLICATION :

FILED: HEREWITH :

FOR: RAFFINOSE SYNTHASE GENE, :
METHOD FOR PRODUCING
RAFFINOSE, AND TRANSGENIC :
PLANT

PRELIMINARY AMENDMENT

ASSISTANT COMMISSIONER FOR PATENTS
WASHINGTON, D.C. 20231

SIR:

Prior to examination on the merits, please amend the above-identified application as follows.

IN THE SPECIFICATION

Please amend the specification as follows.

Page 1, after the title, please insert:

--This application is a Divisional of U.S. Application Serial No. 08/846,234, filed

April 28, 1997, now allowed.--.

Page 10, line 17, replace "57 to 2408" with --56 to 2407--;

line 22, replace "57 to 2408" with --56 to 2407--.

Please delete the original Sequence Listing at pages 66-78.

After the Abstract at page 83, please insert the attached substitute Sequence Listing.

IN THE CLAIMS

Please cancel Claims 1-12.

Please add the following claims.

--13. An isolated DNA encoding a raffinose synthase having the following properties:

- (1) action and substrate specificity: the raffinose synthase produces raffinose from sucrose and galactinol;
- (2) optimum pH: the raffinose synthase has an optimum pH of about 6 to 8;
- (3) optimum temperature: the raffinose synthase has an optimum temperature of about 35 to 40°C;
- (4) molecular weight: the raffinose synthase has:
 - (i) a molecular weight of about 75 kDa to 95 kDa estimated by gel filtration chromatography;
 - (ii) a molecular weight of about 90 kDa to 100 kDa estimated by polyacrylamide gel electrophoresis; and
 - (iii) a molecular weight of about 90 kDa to 100 kDa estimated by SDS-polyacrylamide gel electrophoresis under a reduced condition; and
- (5) inhibition: the raffinose synthase is inhibited by iodoacetamide, N-ethylmaleimide, and myo-inositol.

14. The DNA of Claim 13, which wherein the raffinose synthase comprises an amino acid sequence shown in SEQ ID NO: 1, 2 or 3.

15. An isolated DNA encoding a raffinose synthase, wherein the DNA is hybridizable under stringent conditions to a DNA comprising nucleotide numbers 56 to 2407 of SEQ ID NO: 4.

16. The DNA of Claim 15, wherein the stringent conditions are 0.1 x SSC, 0.1% SDS at 60°C.

17. An isolated DNA encoding a raffinose synthase having a homology of not less than 35% with respect to the raffinose synthase shown in SEQ ID NO: 5.

18. The DNA of Claim 17, wherein the raffinose synthase has a homology of not less than 40% with respect to the raffinose synthase shown in SEQ ID NO: 5.

19. The DNA of Claim 17, wherein the raffinose synthase has a homology of 65% in the region between the 510th and 610th amino acid of SEQ ID NO: 5.

20. An isolated DNA encoding a raffinose synthase, wherein the DNA has a homology of not less than about 50% with respect to the nucleotide sequence of SEQ ID NO: 4.

21. An isolated DNA encoding a raffinose synthase, wherein the DNA has a homology of not less than about 65% with respect to a region comprising about 300 nucleotide residues in the nucleotide sequence of SEQ ID NO: 4.

22. An isolated DNA encoding a raffinose synthase, wherein the DNA is obtained from a dicotyledonous plant.

23. The DNA of Claim 22, wherein the dicotyledonous plant is a *Cucurbitaceae* *Leguminosae* or plant

24. The DNA of Claim 22, wherein the dicotyledonous plant is a *Cucurbitaceae* plant.

25. The DNA of Claim 24, wherein the *Cucurbitaceae* plant is a melon or a cucumber.
26. The DNA of Claim 24, wherein the *Cucurbitaceae* plant is *Cucumis melo* or *Cucumis sativus*.
27. An isolated DNA encoding a raffinose synthase, wherein the DNA is obtained by a process comprising screening a cDNA library isolated from cucumber by hybridization with an oligonucleotide probe, wherein the oligonucleotide probe encodes a partial amino acid sequence of SEQ ID NO: 5.
28. The DNA of Claim 15, wherein the raffinose synthase has a homology of not less than 35% with respect to the raffinose synthase shown in SEQ ID NO: 5.
29. The DNA of Claim 15, wherein the raffinose synthase has a homology of not less than 40% with respect to the raffinose synthase shown in SEQ ID NO: 5.
30. The DNA of Claim 15, wherein the raffinose synthase has a homology of not less than 65% in the region between the 510th and 610th amino acid of SEQ ID NO: 5.
31. The DNA of Claim 13, wherein the DNA is hybridizable under stringent conditions to a DNA comprising nucleotide numbers 56 to 2407 of SEQ ID NO: 4.
32. The DNA of Claim 13, wherein the stringent conditions are 1 x SSC, 0.1% SDS at 60°C.
33. The DNA of Claim 13, wherein the stringent conditions are 0.1 x SSC, 0.1% SDS at 60°C.
34. The DNA of Claim 13, wherein the raffinose synthase has a homology of not less than 35% with respect to the raffinose synthase shown in SEQ ID NO: 5.
35. The DNA of Claim 13, wherein the raffinose synthase has a homology of not less than 40% with respect to the raffinose synthase shown in SEQ ID NO: 5.

36. The DNA of Claim 13, wherein the raffinose synthase has a homology of not less than 65% in the region between the 510th and 610th amino acid of SEQ ID NO: 5.--

SUPPORT FOR THE AMENDMENTS

Page 1 of the specification has been amended to insert a reference to the parent application. Page 10 of the specification has been amended to correct an error identifying the coding sequence in SEQ ID NO: 4. This correction is supported by the entry for SEQ ID NO: 4 in the original Sequence Listing at page 67. Newly added Claims 13-36 are supported by the specification at pages 7-76 and by original Claims 1-82. Support for the recitation of dicotyledenous plant in Claim 22 is provided by the fact that the plant disclosed in the present application are dicotyledonous. Support for the recitation of *Leguminosae* in Claim 23 is provided from the Description of the Background Art in the specification at pages 1-7, which clarifies that the dicotyledonous plant such as the *Leguminosae* plant is useable like the Cucurbitaceae plant. No new matter is believed to have been added to this application by these amendments.

REMARKS

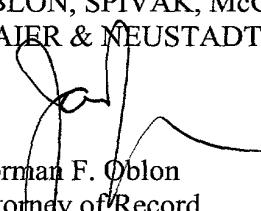
Claims 13-36 are active in this application.

This application is a Divisional of U.S. Application Serial No. 08/846,234, filed April 28, 1997, now allowed.

A substitute Sequence Listing has been submitted. In lieu of submission of a corresponding computer-readable Sequence Listing, Applicants request that the Office use the CRF filed in the parent application on November 15, 1999. Applicants confirm that the sequence information in the substitute Sequence Listing submitted herewith is the same as the CRF submitted on November 15, 1999 in the parent application.

Applicants submit that the present application is ready for examination on the merits. Early notice to this effect is earnestly solicited.

Respectfully submitted,

OBLON, SPIVAK, McCLELLAND,
MAJER & NEUSTADT, P.C.


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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of

Chieko OSUMI, et al.

Serial No. 08/846,234

Filed: April 28, 1997

For: RAFFINOSE SYNTHASE GENE, METHOD FOR PRODUCING
RAFFINOSE, AND TRANSGENIC PLANT

VERIFICATION OF TRANSLATION

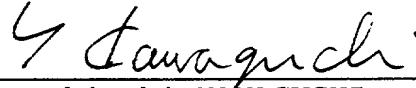
Honorable Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

I, Yoshiyuki KAWAGUCHI, of c/o TOYAMA, MATSUKURA,
KAWAGUCHI & ONO, Yokoyama Building 6th Floor, 4-10, Higashi
Nihonbashi 3-chome, Chuo-ku, Tokyo, 103 Japan, declare:-

- (1) that I know well both Japanese and English languages;
- (2) that I translated the attached Text of Specification
and Claims from Japanese into English;
- (3) that the attached English translation is a true and
correct translation of the Japanese text of specification and
claims as filed in the United States Patent and Trademark
Office on April 28, 1997 under Serial No. 08/846,234 to the
best of my knowledge and belief; and
- (4) that all statements made of my own knowledge are true
and that all statements made on information and belief are
believed to be true, and further that these statements are
made with the knowledge that willful false statements and the
like are punishable by fine or imprisonment, or both, under 18
USC 1001, and that such false statements may jeopardize the
validity of the application or any patent issuing thereon.

Signed at Tokyo, Japan, this 1st day of September, 1997.



Yoshiyuki KAWAGUCHI

RAFFINOSE SYNTHASE GENE, METHOD FOR PRODUCING RAFFINOSE,
AND TRANSGENIC PLANT

Technical Field

The present invention relates to a raffinose synthase, a method for raffinose synthesis based on the use of raffinose synthase or a cell-free extract containing the raffinose synthase, DNA coding for the raffinose synthase, and methods for its use to produce altered amount of raffinose family oligosaccharides in transformed plants. Raffinose is utilized in a variety of fields, as a food material having an activity to proliferate Bifidobacterium, or as a pharmaceutical to be used, for example, for solutions of organ preservation.

15

Background Art

Raffinose is one of raffinose family oligosaccharides, in which galactose is connected to glucosyl group of sucrose via α -1,6 linkage. The raffinose family oligosaccharides include, for example, stachyose containing two connected galactose residues, and verbascose containing three connected galactose residues, in addition to raffinose. These oligosaccharides are widely distributed in plants, for

example, seeds of various plants such as beans, rapeseed, and cottonseed containing these oligosaccharides as reserve carbohydrates; plants belonging to Cucurbitaceae such as cucumber and melon 5 containing these sugars as transport sugars; and sugar beet (Beta vulgaris) and rosette leaves having acquired cold resistance.

The raffinose family oligosaccharides are biosynthesized as follows.

- 10 UDP-galactose + myo-inositol → galactinol + UDP ... (a)
galactinol + sucrose → raffinose + myo-inositol ... (b)
galactinol + raffinose → stachyose + myo-inositol ... (c)

The respective reactions are catalyzed by (a) galactinol synthase (GS: EC 2.4.1.123), (b) raffinose synthase (RS: EC 2.4.1.82), and (c) stachyose synthase (STS: EC 2.4.1.67).

At present, raffinose is extracted from sugar beet, and it is separated and purified in the sucrose purification process. However, since crystal formation 20 of sucrose is deteriorated by raffinose, sugar beet has been subjected to breeding and improvement with the aim of decreasing the raffinose content. As a result, the raffinose content in sugar beet now has a low value of 0.03 % to 0.16 % (Enzyme Microb. Technol., Vol. 4, May, 25 130-135 (1982)). Therefore, it is not easy to efficiently obtain raffinose from sugar beet having such a low raffinose content.

As described above, raffinose is contained in mature seeds of plants belonging to Leguminosae represented by soybean. Mature seed of soybean contains, as soybean oligosaccharides, sucrose (content: about 5 %), stachyose (content: about 4 %), and raffinose (content: about 1 %). The soybean oligosaccharides are recovered in a fraction obtained by deproteinizing defatted soybean, and they are utilized, for example, for functional food products after concentration. However, raffinose occupies a proportion of 10 % of the whole oligosaccharides, and hence raffinose exists in a small amount.

On the other hand, a method for enzymatically synthesizing raffinose has been reported (Trends in Glycoscience and Glycotechnology, 7.34, 149-158 (1995)). This method comprises the steps of synthesizing galactobiose in accordance with a condensation reaction catalyzed by α -galactosidase, and transferring galactosyl group to sucrose by using the galactobiose as a galactosyl group donor in accordance with a galactosyl transfer reaction to synthesize raffinose. However, in this reaction, 350 g of galactobiose is synthesized from 1.9 kg of lactose hydrolysate, and 100 g of raffinose is obtained from 190 g of galactobiose and 760 g of sucrose. Therefore, the yield of produced raffinose is low, and hence this synthesis method is not efficient.

Besides the foregoing methods, a method is also

conceivable in which a plant having a high raffinose content may be bred by means of transformation for genes for enzymes included in the biosynthesis system. For example, Kerr et al. have cloned a gene for galactinol synthase, and transformed rapeseed therewith (WO 5 93/02196). As a result, the GS activity was increased, however, the content of the raffinose family oligosaccharides was unwillingly decreased. It was impossible to achieve the object to enhance the 10 biosynthesis of the raffinose family oligosaccharides by transforming the galactinol synthase gene. Therefore, there has not been provided a method for increasing the content of the raffinose family oligosaccharides in plant.

15 On the other hand, it is also demanded to decrease the raffinose family oligosaccharides. As described above, the raffinose family oligosaccharides are widely distributed over plants including, seeds of various plants such as beans, for example, soybean, rapeseed, 20 and cottonseed containing these oligosaccharides as storage carbohydrates; plants belonging to Cucurbitaceae such as cucumber and melon containing these oligosaccharides as transport sugars; and sugar beet and rosette leaves having acquired cold resistance. Meals 25 obtained after extraction of oil, for example, from soybean, rapeseed, and cotton contain the raffinose family oligosaccharides. Almost all of the meals are

utilized as feed. However, human and animals, which do not have α -galactosidase, cannot directly digest the raffinose family oligosaccharides. It is known that the raffinose family oligosaccharides lower the metabolic energy efficiency of feed due to, for example, assimilation of the raffinose family oligosaccharides by enteric bacteria to cause gas production. It has been reported that removal of raffinose family oligosaccharides from soybean meal results in a large increase in the metabolizable energy for broiler chickens (Coon, "Proceeding Soybean Utilization Alternatives", Univrsity of Minnesota, 203-211 (1989)). In view of the foregoing facts, it is desired to develop the plants such as soybean, rapeseed, and cottonseed in which the raffinose family oligosaccharides are decreased.

Such plants have been subjected to breeding to increase the amount of oil. Photosynthetic products are distributed over oils, proteins, and carbohydrates including the raffinose family oligosaccharides. It has been reported for soybean that a reverse correlation exists between the amount of oils and the amount of carbohydrates. It is expected that the content of oils can be increased in a soybean plant having the same photosynthetic ability as those possessed by others, by decreasing the production of the raffinose family oligosaccharides.

Based on a viewpoint as described above, Kerr et al. have reported development of soybean varieties with a low content of the raffinose family oligosaccharides, by means of breeding based on mating and selection, in 5 which the raffinose family oligosaccharides are lowered by an amount of 80 % to 90 % (WO 93/00742). However, this technique concerns creation of soybean variety, which cannot be applied to other various soybean varieties developed in response to, for example, 10 aptitude for cultivation and resistance to disease. This technique cannot be universally applied to various plants as well.

It is known that raffinose, which is contained, for example, in sugar beet and sugar cane, lowers crystal 15 formation of sugar or sucrose. Therefore, it is possible to expect that if no raffinose is produced, the production efficiency of sugar may be improved in such a plant. However, no sugar beet has been created, which contains no raffinose.

20 As described above, the raffinose synthase, which has been hitherto purified, has been confirmed only as an enzyme activity, and no entity of the enzyme has been identified. The confirmed activity is low, and it has been desired to obtain a raffinose synthase having a 25 high activity. The conventional method for producing raffinose provides a low yield, and hence it has been desired to develop an efficient method for producing

raffinose. On the other hand, it is also desired to breed a plant in which the raffinose family oligosaccharides are decreased.

Disclosure of the Invention

5 The present invention has been made taking the foregoing viewpoints into consideration, an object of which is to obtain a raffinose synthase having a high activity and DNA encoding raffinose synthase, and provide an efficient method for enzymatically synthesis
10 raffinose, and a method for utilizing DNA encoding raffinose synthase in plants.

As a result of diligent investigations in order to achieve the object described above, the present inventors have succeeded in purifying a raffinose synthase from cucumber. Further diligent investigations have been made by the present inventors in order to clone a gene coding for the raffinose synthase. As a result, a DNA fragment specific to a gene for the raffinose synthase has been obtained by chemically synthesizing single strand DNA's on the basis of nucleotide sequences deduced from amino acid sequences of peptide fragments of the cucumber raffinose synthase, and performing PCR by using the single strand synthetic DNA's as primers and using cDNA's prepared from poly(A)⁺RNA extracted from cucumber as templates.
20
25

Further, the raffinose synthase gene has been isolated by adopting a method in which hybridization is performed for a cDNA library originating from cucumber by using the DNA fragment as a probe. A chimeric gene having a regulatory region expressible in plants has been prepared by using a fragment of the isolated raffinose synthase gene to transform a plant. Further, the function of endogenous raffinose synthase has been regulated by introducing the raffinose synthase gene to create a plant in which the raffinose family oligosaccharides are decreased.

Namely, the present invention provides a raffinose synthase which has the following properties:

- (1) action and substrate specificity: the raffinose synthase produces raffinose from sucrose and galactinol;
- (2) optimum pH: the raffinose synthase has an optimum pH of about 6 to 8;
- (3) optimum temperature: the raffinose synthase has an optimum temperature of about 35 to 40 °C;
- (4) molecular weight: the raffinose synthase has:
 - (i) a molecular weight of about 75 kDa to 95 kDa estimated by gel filtration chromatography;
 - (ii) a molecular weight of about 90 kDa to 100 kDa estimated by polyacrylamide gel electrophoresis (Native PAGE); and
 - (iii) a molecular weight of about 90 kDa to 100 kDa

estimated by SDS-polyacrylamide gel electrophoresis
(SDS-PAGE) under a reduced condition;

(5) inhibition: the raffinose synthase is inhibited by iodoacetamide, N-ethylmaleimide, and myo-
5 inositol.

In a specified embodiment of the foregoing raffinose synthase provided by the present invention, the raffinose synthase has an amino acid sequence including respective amino acid sequences shown in SEQ 10 ID NOs. 1 to 3 in Sequence Listing.

In another aspect of the present invention, there is provided a raffinose synthase which is a protein specified by the following item (A) or (B):

(A) a protein which has an amino acid sequence 15 shown in SEQ ID NO: 5 in Sequence Listing; or
(B) a protein which comprises an amino acid sequence including substitution, deletion, insertion, addition, or inversion of one or several residues of amino acids in the amino acid sequence shown in SEQ ID 20 NO: 5 in Sequence Listing, and which has an activity to produce raffinose from sucrose and galactinol.

In still another aspect of the present invention, there is provided a method for producing raffinose, comprising the step of allowing the foregoing raffinose 25 synthase to act on sucrose and galactinol to produce raffinose.

In still another aspect of the present invention,

there are provided DNA encoding raffinose synthase, and DNA coding for a protein specified by the following item (A) or (B):

- (A) a protein which has an amino acid sequence
5 shown in SEQ ID NO: 5 in Sequence Listing; or
(B) a protein which comprises an amino acid sequence including substitution, deletion, insertion, addition, or inversion of one or several residues of 10 amino acids in the amino acid sequence shown in SEQ ID NO: 5 in Sequence Listing, and which has an activity to produce raffinose from sucrose and galactinol.

In a specified embodiment of the foregoing DNA of the present invention, there is provided DNA specified by the following item (a) or (b):

- 15 (a) DNA which includes a nucleotide sequence comprising at least nucleotide residues having nucleotide numbers of 57 to 2408 in a nucleotide sequence shown in SEQ ID NO: 4 in Sequence Listing; or
(b) DNA which is hybridizable under a stringent condition with the nucleotide sequence comprising at 20 least nucleotide residues having nucleotide numbers of 57 to 2408 in the nucleotide sequence shown in SEQ ID NO: 4 in Sequence Listing, and which codes for a protein having an activity to produce raffinose from sucrose and 25 galactinol.

In still another aspect of the present invention, there are provided a chimeric gene comprising a

raffinose synthase gene or a part thereof, and a transcription regulatory region expressible in plant cells, and a plant transformed with the chimeric gene.

In still another aspect of the present invention,
5 there is provided a method for changing a content of raffinose family oligosaccharides in a plant, comprising the steps of transforming the plant with the chimeric gene, and expressing the gene in the plant.

In the following description, the raffinose
10 synthase having the properties described in the foregoing items (1) to (5), or the raffinose synthase specified as the protein defined in the foregoing items (A) and (B) is simply referred to as "raffinose synthase" in some cases. DNA encoding raffinose
15 synthase, or DNA encoding raffinose synthase and including non-translating regions is referred to as "raffinose synthase gene" in some cases.

The present invention will be explained in detail below.

20 <1> Raffinose synthase of the present invention

The raffinose synthase of the present invention has the following properties:

- (1) action and substrate specificity: the raffinose synthase produces raffinose from sucrose and
25 galactinol;
- (2) optimum pH: the raffinose synthase has an

optimum pH of about 6 to 8;

(3) optimum temperature: the raffinose synthase has an optimum temperature of about 35 to 40 °C;

5 (4) molecular weight: the raffinose synthase has:
(i) a molecular weight of about 75 kDa to 95 kDa estimated by gel filtration chromatography;

(ii) a molecular weight of about 90 kDa to 100 kDa estimated by polyacrylamide gel electrophoresis (Native PAGE); and

10 (iii) a molecular weight of about 90 kDa to 100 kDa estimated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under a reduced condition;

15 (5) inhibition: the raffinose synthase is inhibited by iodoacetamide, N-ethylmaleimide, and myo-
inositol.

The raffinose synthase having the foregoing properties has been isolated and purified from leaves of cucumber, which has been identified for the first time by the present inventors. As demonstrated in Examples described later on, the raffinose synthase originating from cucumber includes the respective amino acid residues shown in SEQ ID NOS: 1 to 3 in Sequence Listing, in the amino acid sequence of the enzyme protein. An entire amino acid sequence of the raffinose synthase is shown in SEQ ID NO: 5.

The raffinose synthase is obtainable from plants belonging to Cucurbitaceae, for example, plants such as

melon (Cucumis melo) and cucumber (Cucumis sativus).

Especially, the raffinose synthase is contained in a large amount in leaves of these plants, especially in tissues of leaf veins and seeds.

5 Next, the method for producing the raffinose synthase of the present invention will be explained in accordance with an illustrative method for isolating and purifying the raffinose synthase from cucumber.

Leaf vein tissues are collected from leaves of
10 cucumber obtained 6 to 10 weeks after planting. The vein are ground by a mortar with liquid nitrogen, to which a buffer is added to extract proteins. During this process, it is allowable to add a substance to prevent the raffinose synthase from degradation and
15 inactivation, for example, a protease inhibitor such as PMSF (phenylmethanesulfonyl fluoride), or polyclarl AT (produced by Serva). Insoluble matters are removed from an obtained extract solution by means of filtration and centrifugation to obtain a crude extract solution.

20 The crude extract solution thus obtained is subjected to fractionation based on combination of ordinary methods for purifying proteins, including, for example, anion exchange chromatography, hydroxyapatite chromatography, gel filtration, and salting out. Thus
25 the raffinose synthase can be purified.

Anion exchange chromatography can be performed, for example, by using a column charged with a strongly basic

anion exchanger such as HiTrap Q (produced by Pharmacia), or a weakly basic anion exchanger such as DEAE-TOYOPEARL (produced by Toyo Soda). The extract solution containing the raffinose synthase is allowed to 5 pass through the column so that the enzyme is adsorbed to the column. After washing the column, the enzyme is eluted by using a buffer having a high salt concentration. During this process, the salt concentration may be increased in a stepwise manner, or 10 the concentration gradient may be applied. For example, when the HiTrap Q column is used, the raffinose synthase activity adsorbed to the column is eluted by NaCl at about 0.3 M. An eluting solution to give an NaCl concentration gradient of 0.05 M to 0.35 M is preferably 15 used for DEAE-TOYOPEARL. An eluting solution to give a phosphate concentration gradient of 0.01 M to 0.3 M is preferably used for hydroxyapatite chromatography.

The order of the foregoing operations is not specifically limited. Each of the operations may be 20 repeated two or more times. It is desirable to exchange a sample solution with an appropriate buffer by means of dialysis or the like before the sample solution is allowed to pass through each column. The sample solution may be concentrated at each stage.

25 At each stage of the purification, it is preferable that the raffinose synthase activity contained in each of fractionated fractions is measured so that fractions

having high activities are collected to be used in the next stage. The method for measuring the raffinose synthase activity is exemplified by a method based on the use of radioisotope as reported, for example, by
5 Lehle, H. et al. (Eur. J. Biochem., 38, 103-110 (1973)). As a modified method thereof, the reaction temperature and the substrate concentration may be changed. For example, 10 μ l of an enzyme solution is added to a reaction solution containing, at final concentrations,
10 10 mM ^{14}C -sucrose, 20 mM galactinol, 25 mM HEPES (2-(4-(2-hydroxyethyl)-1-piperazinyl)ethanesulfonic acid)-NaOH, pH 7.0, 0.5 mM DTT (dithiothreitol) to give a volume of 50 μ l. The solution is incubated at 32 °C for 1 hour to perform the reaction. The reaction is stopped
15 by adding 200 μ l of ethanol and heating the solution at 95 °C for 30 seconds. The reaction solution is centrifuged to obtain a supernatant. An aliquot of the supernatant is spotted on Whatman 3MM filter paper, and developed with n-propanol: ethyl acetate: water = 4:1:2.
20 Incorporation of ^{14}C into raffinose is investigated, which is regarded to be the raffinose synthase activity (nmol/hour).

The present inventors have developed a method for measuring the raffinose synthase activity in place of
25 the foregoing method. Namely, the raffinose synthase activity is measured by quantitatively determining raffinose produced by the raffinose synthesis reaction,

by means of HPLC (high-performance liquid chromatography). According to this method, the activity can be measured conveniently and quickly as compared with the method of Lehle, H. et al. This method is
5 especially preferable to detect active fractions during the purification operation. This method will be explained below.

The raffinose synthesis reaction is based on the use of a reaction solution prepared to have a
10 composition having the following final concentrations. The reaction solution is added with 10 to 50 µl of a raffinose synthase solution to give a volume of 100 µl, followed by performing the reaction at 32 °C for 60 minutes.

15 [Composition of reaction solution (final concentration)]
2.5 mM sucrose
5 mM galactinol
5 mM DTT
20 mM Tris-HCl buffer (pH 7.0)

20 After performing the reaction as described above, the reaction solution is added with ethanol in a volume four times the volume of the reaction solution to stop the reaction by heating the solution at 95 °C for 30 seconds. The obtained solution is centrifuged to obtain
25 a supernatant which is then dried up under a reduced

pressure. After that, an obtained residue is dissolved in distilled water. Raffinose in the reaction product is quantitatively determined by using HPLC to estimate the raffinose synthase activity. HPLC can be performed 5 by using, for example, Sugar Analysis System DX500 (CarboPac PA1 column, pulsed amperometry detector (produced by Dionex)).

Fig. 1 shows a result of measurement performed in accordance with the method described above, for the 10 amount of raffinose produced when the reaction time was changed. As clarified from Fig. 1, this method makes it possible to conveniently measure the raffinose synthase activity with excellent linearity.

The degree of purification of the purified 15 raffinose synthase can be confirmed, and the molecular weight can be measured, by means of, for example, gel electrophoresis and gel filtration chromatography. Enzymatic properties can be investigated by measuring the enzyme activity while changing the reaction 20 temperature or the reaction pH, or by measuring the remaining enzyme activity after adding, to the reaction solution, various enzyme inhibitors, metal ions or the like. The stable pH range and the stable temperature range can be investigated by measuring the enzyme 25 activity after exposing the raffinose synthase to various pH conditions and temperature conditions for a certain period of time respectively.

The properties of the raffinose synthase described above have been determined in accordance with procedures as described above. However, it should be noted that different results may be obtained depending on measurement conditions. For example, the measurement for the molecular weight based on the use of gel filtration chromatography is affected by the type of the gel filtration agent and the buffer, and the molecular weight marker to be used. The enzyme activity differs depending on the type of the buffer and the salt concentration in many cases even when the measurement is performed at an identical pH. Therefore, upon identification for the raffinose synthase, it is preferable to perform comprehensive investigation without being bound to only measurement for individual properties.

The raffinose synthase of the present invention is obtained by performing the isolation and purification from cucumber as described above. Alternatively, the raffinose synthase of the present invention can be produced by introducing, into an appropriate host, DNA coding for the raffinose synthase described later on, and making expression thereof, in accordance with ordinary methods used for fermentative production of heterogeneous proteins.

Those assumed as the host for expressing the raffinose synthase gene include various prokaryotic

cells represented by Escherichia coli, and various eucaryotic cells represented by Saccharomyces cerevisiae. However, it is desirable to use plant cells, especially cells originating from plants such as 5 tobacco, cucumber, and Arabidopsis thaliana.

The recombinant plasmid used for transformation can be prepared by inserting DNA coding for the raffinose synthase into an expression vector in conformity with the type of cells to be used for expression therein. 10 Those usable as the plant expression vector include those having a promoter DNA sequence capable of being expressed in the plant or a combination of a plurality of such promoter DNA sequences, and a terminator DNA sequence workable in the plant, and further having a 15 sequence between the both to make it possible to insert a foreign gene.

The promoter includes, for example, promoters which make expression over a whole plant, such as CaMV 35S RNA promoter, CaMV 19S RNA promoter, and nopaline synthase promoter; promoters which make expression in green tissues, such as Rubisco small subunit promoter; and promoters which make site-specific expression at portions such as seed, including, for example, those for genes of napin and phaseolin. The terminator described 20 above includes, for example, nopaline synthase terminator, and Rubisco small subunit 3'-side portion. 25

As for the expression vector for plants, for

example, pBI121 and p35S-GFP (produced by CLONTECH) are commercially available, which may be used.

Alternatively, a vector for expressing virus RNA may be used so that a gene for an outer coat protein encoded 5 thereby, for example, may be replaced with the raffinose synthase gene.

In order to achieve transformation, it is advantageous to use methods which are usually used for transformation, such as the Agrobacterium method, the 10 particle gun method, the electroporation method, and the PEG method, in conformity with a host cell to be manipulated. The raffinose synthase activity can be detected by using the method adopted in the purification process for the raffinose synthase. Upon the detection, 15 it is desirable to previously remove α -galactosidase, for example, by allowing the sample to pass through an anion exchange column.

The gene coding for the raffinose synthase originating from cucumber includes all of those which 20 provide the raffinose synthase activity upon expression. Preferably, the gene is exemplified by the gene comprising DNA coding for the amino acid sequence shown in SEQ ID NO: 5 in Sequence Listing, and the gene having the nucleotide sequence shown in SEQ ID NO: 4 in 25 Sequence Listing. It is noted that the gene coding for the amino acid sequence shown in SEQ ID NO: 5 in Sequence Listing includes various nucleotide sequences

taking degeneracy of codons into consideration. Namely, the gene coding for the amino acid sequence shown in SEQ ID NO: 5 in Sequence Listing may be selected from such various nucleotide sequences, while considering several factors for the gene expression system, such as preferential codons depending on, for example, the type of the host cell, and avoidance of higher-order structure to be formed by transcribed RNA. The selected nucleotide sequence may be DNA cloned from the nature, or DNA chemically synthesized in an artificial manner.

<2> DNA coding for raffinose synthase of the present invention

DNA coding for the raffinose synthase can be obtained by preparing a cDNA library from poly(A)⁺RNA isolated from a plant such as cucumber, and screening the cDNA library by means of hybridization. A probe to be used for the hybridization can be obtained by performing amplification by means of PCR (polymerase chain reaction) by using, as primers, oligonucleotides synthesized on the basis of partial amino acid sequences of the raffinose synthase protein.

A method for obtaining DNA of the present invention from poly(A)⁺RNA originating from cucumber will be specifically explained below.

As for the position for extracting poly(A)⁺RNA, all portions of a cucumber plant body may be used

provided that the raffinose synthase gene is expressed at that portion. poly(A)⁺RNA can be obtained, for example, from leaves, stalks, buds, fruits, and seeds at various growth stages. However, poly(A)⁺RNA is
5 desirably obtained from a material of fully expanded leaves after fruiting, especially leaf vein portions.

In order to extract total RNA from the cucumber tissue, any method may be used without limitation provided that RNA can be efficiently obtained with less
10 damage. It is possible to use any known method such as the phenol/SDS method and the guanidine isothiocyanate/cesium chloride method. Poly(A)⁺RNA can be isolated from the total RNA thus obtained, by using an oligo(dT) cellulose. It is also preferable to use a
15 kit (for example, MPG Direct mRNA Purification Kit, produced by CPG, INC.) which makes it possible to obtain poly(A)⁺RNA without extracting the total RNA.

A DNA fragment, which is used as a probe for screening for the cDNA library, can be obtained by
20 performing PCR. Oligonucleotides, which have nucleotide sequences deduced from already known amino acid sequences of peptide fragments, for example, nucleotide sequences deduced from amino acid sequences shown in SEQ ID NOS: 1 to 3, are chemically synthesized. The
25 obtained oligonucleotides are used as primers to perform PCR. Any portion of the amino acid sequence of the obtained peptide fragment may be used for the primers.

However, it is desirable to select sequences which include less degeneracy of codons and which are assumed to form no complicated higher-order structure.

Alternatively, it is also preferable to perform RACE
5 (Rapid Amplification of cDNA End, "PCR PROTOCOLS A Guide to Methods and Applications", ACADEMIC press INC., pp. 28 to 38).

It is desirable to use, as a template for PCR, a cDNA library or single strand cDNA. When heat-resistant
10 DNA polymerase having a reverse transcriptase activity is used for the PCR reaction, it is allowable to use poly(A)⁺RNA, or total RNA in some cases.

In order to prepare the cDNA library, at first single strand cDNA's are synthesized by using reverse
15 transcriptase while using poly(A)⁺RNA as a template and using oligo(dT) primer and random primers. Next, double strand cDNA's are synthesized in accordance with, for example, the Gubler and Hoffman method, the Okayama-Berg method ("Molecular Cloning", 2nd edition, Cold Spring Harbor press, 1989). When the raffinose synthase gene is expressed in a small amount, cDNA's may be amplified by means of PCR by using a cDNA library construction kit based on the use of PCR (for example, Capfinder PCR cDNA Library Construction Kit (produced by CLONTECH)).
20 cDNA's thus synthesized can be cloned into a cloning vector such as phage vectors and plasmids, after performing, for example, blunt end formation, addition
25

of linker, addition of restriction enzyme site by means of PCR.

A portion characteristic of the raffinose synthase cDNA is selected from the DNA fragments obtained by PCR described above, for the probe for hybridization. It is desirable to select a DNA fragment located near to the 5'-terminal side. The amplified DNA fragment thus selected is purified from a reaction solution of PCR. In this procedure, the amplified DNA fragment may be purified by subcloning the DNA fragment by using a plasmid, preparing a large amount of a subcloned plasmid which is thereafter digested with a restriction enzyme, and excising the DNA fragment from a gel after electrophoresis. Alternatively, the amplified DNA fragment may be purified by performing PCR by using the plasmid as a template to amplify and use only the objective portion. When the amount of the initially amplified DNA fragment is sufficiently large, the amplified DNA fragment may be purified by electrophoresing the DNA fragment without performing subcloning, excising a gel segment containing a band of the objective DNA fragment, and purifying the DNA fragment from the gel segment.

Screening to obtain the objective clone from the cDNA library is performed by means of hybridization. The DNA fragment obtained in accordance with the foregoing method can be labeled and used as a probe for

the hybridization. Upon labeling, it is possible to use various labels such as radioisotope and biotin. However, labeling is desirably performed in accordance with the random priming method. Screening may be 5 performed by using PCR instead of hybridization. Further, screening may be performed by using hybridization and PCR in combination.

The nucleotide sequence of DNA coding for the raffinose synthase originating from cucumber obtained as 10 described above, and the amino acid sequence deduced from the nucleotide sequence are illustratively shown in SEQ ID NO: 4 in Sequence Listing. Only the amino acid sequence is shown in SEQ ID NO: 5. A transformant AJ13263 of Escherichia coli JM109, which harbors a 15 plasmid pMossloxCRS containing the DNA fragment including DNA coding for the raffinose synthase obtained in Example 3 described later on, has been internationally deposited on the basis of the Budapest Treaty since November 19, 1996 in National Institute of 20 Bioscience and Human Technology of Agency of Industrial Science and Technology of Ministry of International Trade and Industry (postal code: 305, 1-3 Higashi-Icchome, Tsukuba-shi, Ibaraki-ken, Japan), and awarded a deposition number of FERM BP-5748.

25 The DNA of the present invention may code for a raffinose synthase protein including substitution, deletion, insertion, addition, or inversion of one or

several residues of amino acids at one or several positions, provided that the activity of raffinose synthase encoded thereby, i.e., the activity to produce raffinose from sucrose and galactinol is not

5 deteriorated. In this context, the number of "several residues" differs depending on the position and the type of the amino acid residues in the three-dimensional structure of the protein, originally because of the following reason. Namely, high similarity is found

10 between some amino acids and other amino acids, for example, between isoleucine and valine, and such a difference in amino acid does not greatly affect the three-dimensional structure of the protein. Therefore, the DNA of the present invention may code for those

15 having homology of not less than 35 to 40 % with respect to the entire 784 amino acid residues for constructing the raffinose synthase originating from cucumber, provided that they have the raffinose synthase activity. Preferably, those encoded by the DNA of the present

20 invention have homology of 65 % in a region between 510th amino acid and 610th amino acid. Specifically, the number of "several residues" is 2 to 40, preferably 2 to 20, and more preferably 2 to 10.

The present invention includes genes in which

25 homology of not less than about 50 % is given for the entire length of the gene, and homology of not less than 65 % is given over a region comprising about 300

nucleotide residues. Nucleotide sequence information on such genes can be obtained by searching genes having homology to the raffinose synthase gene originating from cucumber, by using data base such as GenBank. For example, GENETIX-MAC (software for processing genetic information, produced by Software Development), which adopts the Lipman-Person method, may be used as a program for homology analysis. Alternatively, those open to the public on the Internet may be used for this purpose. Some nucleotides sequences obtained by the method as described above contain the entire length of the gene, and other nucleotide sequences do not contain the entire length of the gene. When the entire length of the gene is not contained, the entire length gene can be easily obtained by using RNA extracted from an objective plant tissue as a template, and using primers corresponding to portions having high homology to the raffinose synthase gene originating from cucumber, in accordance with the 5'-RACE method and the 3'-RACE method. The obtained entire length gene may be incorporated into an appropriate expression vector provided as those included in a kit such as Soluble Protein Expression System (produced by INVITROGEN), Tight Control Expression System (produced by INVITROGEN), and QIAexpress System (produced by QIAGEN) so that the gene may be expressed. The raffinose synthase activity may be measured in accordance with the

method described above to select a clone having the activity.

DNA, which codes for substantially the same protein as the raffinose synthase, is obtained by modifying the 5 nucleotide sequence in accordance with, for example, the site-directed mutagenesis method so that amino acids located at specified positions are subjected to substitution, deletion, insertion, or addition.

Modified DAN as described above may be also obtained in 10 accordance with the conventionally known mutation treatment. The mutation treatment includes a method in which the DNA coding for the raffinose synthase is treated with hydroxylamine or the like in vitro, and a method in which a bacterium belonging to the genus 15 Escherichia harboring the DNA coding for the raffinose synthase gene is treated with ultraviolet irradiation or a mutating agent usually used for artificial mutation, such as nitrous acid and N-methyl-N'-nitro-N-nitrosoguanidine (NTG).

20 The substitution, deletion, insertion, addition, or inversion of the nucleotide includes mutation which naturally occurs, for example, based on the difference between individuals of a cucumber plant, the difference between varieties, the formation of multiple copies of 25 the gene, the difference between respective organs, and the difference between respective tissues.

DNA having mutation as described above is expressed

in an appropriate cell to investigate the raffinose synthase activity of an expressed product. Thus it is possible to obtain DNA which codes for substantially the same protein as the raffinose synthase. Further, DNA 5 coding for substantially the same protein as the raffinose synthase protein can be obtained by isolating DNA which is hybridizable under a stringent condition with DNA having a nucleotide sequence comprising nucleotide residues having, for example, nucleotide 10 numbers of 56 to 2407 in the nucleotide sequence shown in SEQ ID NO: 4 in Sequence Listing, and which codes for the protein having the raffinose synthase activity, from DNA's coding for raffinose synthases having mutation or from cells harboring the DNA's. The phrase "stringent 15 condition" referred to herein indicates a condition in which so-called specific hybrid is formed, and nonspecific hybrid is not formed. It is difficult to definitely express this condition by using numerical values. However, for example, this condition includes a 20 condition in which DNA's having high homology, for example, DNA's having homology of not less than 50 % hybridize with each other, while DNA's having homology lower than the above do not hybridize with each other, or a condition in which hybridization is achieved at a 25 salt concentration corresponding to a washing condition for ordinary Southern hybridization, i.e., 1 x SSC, 0.1 % SDS, and preferably 0.1 x SSC, 0.1 % SDS at 60 °C.

Genes, which hybridize under such a condition, include those which contain a stop codon generated at an intermediate position, and those which have lost the activity due to mutation at the active center. However,

5 those having such inconvenient mutation can be easily eliminated by ligating the gene with a commercially available activity expression vector to measure the raffinose synthase activity in accordance with the method described above.

10 When the DNA of the present invention is used to express antisense RNA for the raffinose synthase, it is unnecessary for the DNA to code for any active raffinose synthase. Further, the function of any endogenous gene having homology can be restrained by using sense RNA.

15 In such a case, it is also unnecessary for the DNA to code for any active raffinose synthase. Further, it is unnecessary for the DNA to contain the entire length. Preferably, it is sufficient for the DNA to have about 500 base pairs of an N-terminal side translating region

20 having 60 % of homology.

The method has been explained above, in accordance with which the present inventors have succeeded in cloning the objective cDNA of the raffinose synthase originating from cucumber. However, other than the foregoing, the following methods may be available.

(1) The raffinose synthase originating from cucumber is isolated and purified, and an entire

nucleotide sequence is chemically synthesized on the basis of a determined amino acid sequence or the amino acid sequence shown in SEQ ID NO: 5.

(2) Chromosomal DNA is prepared from a cucumber plant body, and a chromosomal DNA library is prepared by using a plasmid vector or the like. The raffinose synthase gene is obtained from the library by means of hybridization or PCR. It is assumed that the raffinose synthase gene originating from chromosome contains an intron in its coding region. However, DNA divided into several parts by such intron is included in the DNA of the present invention provided that it codes for the raffinose synthase.

(3) Poly(A)⁺RNA is fractionated into fractions in accordance with the molecular weight or the like. The fractions are subjected to an in vitro translation system based on the use of wheat germ or rabbit reticulocyte to determine a fraction containing mRNA coding for a polypeptide having the raffinose synthase activity. An objective cDNA fragment is prepared and obtained from the fraction.

(4) An anti-cucumber raffinose synthase antibody is prepared. Elements of a cDNA library are incorporated into a protein expression vector, and an appropriate host is infected therewith to express proteins encoded by cDNA's. An objective cDNA may be screened by using the foregoing antibody.

(5) Appropriate primers are synthesized on the basis of amino acid sequences of peptide fragments, and a sequence containing the terminal is amplified by means of the RACE method, followed by cloning thereof.

5 <3> Method for producing raffinose of the present invention

In the method for producing raffinose of the present invention, the raffinose synthase is allowed to act on sucrose and galactinol to produce raffinose.

10 When the raffinose synthase is allowed to act on sucrose and galactinol, the galactose residue used for constructing galactinol is transferred to sucrose, and thus raffinose is produced. During this process, myo-inositol used for constructing galactinol is liberated.

15 The raffinose synthase, which is used to produce raffinose, may be an enzyme extracted from a plant body, or an enzyme produced by means of the genetic recombination technique based on the use of the DNA of the present invention.

20 In order to allow the raffinose synthase to act on sucrose and galactinol, the following procedure may be available. Namely, the raffinose synthase or cells having an ability to produce the raffinose synthase are immobilized to a carrier such as alginic acid gel and
25 polyacrylamide gel to prepare immobilized enzyme or immobilized cells. The immobilized enzyme or the

immobilized cells are charged to a column, and a solution containing sucrose and galactinol is allowed to pass through the column. As for the carrier and the method for immobilizing the raffinose synthase or the 5 cells to the carrier, it is possible to adopt materials and methods which are used for ordinary bioreactors.

The raffinose synthesis reaction is performed, for example, by adding the raffinose synthase to a solution such as an aqueous solution or a buffer containing 10 sucrose and galactinol. It is preferable that pH of the solution is adjusted to be within a range of about 6 to 8, especially at about pH 7. The reaction temperature is within a range of about 28 to 42 °C, preferably 35 to 40 °C, especially about 38 °C. The raffinose synthase 15 of the present invention is stable within a range of pH 5 to 8, especially in the vicinity of pH 6. The enzyme of the present invention is stable within a temperature range of not more than about 40 °C.

The enzyme activity of the raffinose synthase of 20 the present invention is inhibited by iodoacetamide, N-ethylmaleimide, MnCl₂, ZnCl₂, and NiCl₂. Therefore, it is desirable that these substances are not contained in the reaction solution.

Preferably, galactinol and sucrose are added to the 25 reaction solution at a concentration of not less than 5 mM of galactinol and a concentration of not less than 1.5 mM of sucrose. The raffinose synthase may be added

to the reaction solution in an amount depending on the amounts of the substrates.

Raffinose is separated from unreacted sucrose and galactinol and from myo-inositol produced by the enzyme 5 reaction, contained in the reaction solution, in accordance with a method including, for example, gel filtration chromatography.

<4> Chimeric gene and transgenic plant of the present invention

10 The chimeric gene of the present invention includes the raffinose synthase gene or a part thereof and the transcription regulatory region expressible in plant cells. The raffinose synthase gene is exemplified by the DNA coding for the raffinose synthase of the present 15 invention described in the foregoing item <2>. When the chimeric gene of the present invention is used as an antisense gene, a non-coding region of the raffinose synthase gene or a part thereof can be used in some cases, besides the DNA coding for the raffinose 20 synthase. The non-coding region includes, for example, sequences indicated by nucleotide numbers of 1 to 55 (5'-non-coding region) and nucleotide numbers of 2407 to 2517 (3'-non-coding region) in SEQ ID NO: 4 in Sequence Listing.

25 When the transcription regulatory region is linked to the DNA coding for the raffinose synthase in the

chimeric gene of the present invention so that mRNA (sense RNA) homologous to the coding strand of the DNA is expressed, plant cells introduced with the chimeric gene express the raffinose synthase, and the content of 5 the raffinose family oligosaccharides is increased. On the other hand, when the transcriptional regulatory region is linked to the DNA so that RNA (antisense RNA) having a sequence complementary to the coding strand of the DNA is expressed, and when the transcription 10 regulatory region is linked to the DNA so that a partial fragment of the raffinose synthase gene, preferably sense RNA for a portion of not less than about 200 base pairs in the upstream coding region is expressed, then the expression of endogenous raffinose synthase is 15 restrained in plant cells introduced with the chimeric gene, and the raffinose family oligosaccharides are decreased.

The content of the raffinose family oligosaccharides in a plant can be changed by 20 transforming the plant with the chimeric gene of the present invention, and expressing the gene in cells of the plant.

Plants to which the present invention is applicable include, for example, oil crops such as soybean, 25 rapeseed, cotton; sugar crops such as sugar beet and sugar cane; and model plants represented by Arabidopsis thaliana.

The transcription regulatory region expressible in plant cells includes, for example, promoters which make expression over a whole plant, such as CaMV 35S RNA promoter, CaMV 19S RNA promoter, and nopaline synthase promoter; promoters which make expression in green tissues, such as Rubisco small subunit promoter; and promoter regions which make site-specific expression at portions such as seed, including, for example, those for genes of napin and phaseolin as described above. The 5 10 15 20 25 3'-terminal of the chimeric gene may be connected with the terminator such as nopaline synthase terminator, and Rubisco small subunit 3'-end portion.

The plant may be transformed with the chimeric gene in accordance with usually used methods such as the Agrobacterium method, the particle gun method, the electroporation method, and the PEG method, depending on the host cell to be manipulated.

The transformation method for introducing the chimeric gene into the plant includes, for example, the Agrobacterium method, the particle gun method, the electroporation method, and the PEG method.

The Agrobacterium method is specifically exemplified by a method based on the use of a binary vector. Namely, a plant is infected with a vector comprising T-DNA originating from Ti plasmid, a replication origin which is functional in microorganisms such as Escherichia coli, and a marker gene for

selecting plant cells or microbial cells harboring the vector. Seeds are collected from the plant, and they are allowed to grow. Plants introduced with the vector are selected by using an index of expression of the 5 marker gene. Obtained plants are measured for the raffinose synthase activity, or strains exhibiting change in content of the raffinose family oligosaccharides are selected from the obtained plants. Thus it is possible to obtain an objective transformed 10 plant.

A method for introducing the chimeric gene into soybean will be described below. In order to perform transformation for soybean, it is possible to use any one of the particle gun method (Pro. Natl. Acad. Sci. USA, 86, 145 (1989); TIBTECH, 8, 145 (1990); Bio/Technology, 6, 923 (1988); Plant Physiol., 87, 671 (1988); Develop. Genetics, 11, 289 (1990); and Plant 15 cell Tissue & Organ Culture, 33, 227 (1993)), the Agrobacterium method (Plant Physiol., 91, 1212 (1989); WO 94/02620; Plant Mol. Biol., 9, 135 (1987); and Bio/Technology, 6, 915 (1983)), and the electroporation 20 method (Plant Physiol., 99, 81 (1992); Plant Physiol., 84, 856 (1989); and Plant Cell Reports, 10, 97 (1991)).

In the particle gun method, it is preferable to use 25 an embryogenic tissue or a hypocotyl of an immature seed about 30 to 40 days after dehiscence of anthesis. About 1 g of the embryogenic tissue is spread over a petri

dish, into which, for example, gold particles or tungsten particles coated with the objective chimeric gene may be shot. The tissue is transferred after 1 to 2 hours to a liquid medium to perform cultivation.

5 After 2 weeks, the tissue is transferred to a medium containing an antibiotic for transformant selection, followed by cultivation. After 6 weeks, a green adventitious embryo which is resistant to the antibiotic is obtained. The adventive embryo is further

10 transferred to a fresh medium and cultured so that a plant body is reproduced. Alternatively, when the hypocotyl is used, the hypocotyl is excised under a sterilized condition, and it is treated in accordance with the particle gun method, followed by cultivation in

15 MS medium (Murashige and Skoog, Physiologia Plantrum, 15, 473-497 (1962)) containing cytokinin at a high concentration. The hypocotyl is cultured in the darkness for 2 weeks, and then it is cultured at room temperature with light irradiation for 12 to 16 hours in

20 MS medium having a lowered cytokinin content. During this process, it is preferable to add, to the medium, the antibiotic having been used as the selection marker. When a multiple bud body is formed from the transplanted tissue, it is transferred to a medium added with no

25 hormone so that rooting is caused. An obtained seedling body is transferred to a greenhouse and cultivated.

In the case of the method based on the use of

Agrobacterium, it is desirable to use Cotyldonary nod as a plant tissue. Commercially available LBA4404, C58, and Z707 can be used as Agrobacterium. However, it is desirable to use Z707. For example, a plasmid obtained by inserting the objective gene into pMON530 (produced by Monsanto Co.) can be used as the vector. The plasmid is introduced into Agrobacterium tumefaciens Z707 (Hepburn et al., J. Gen. Microbiol., 131, 2961 (1985)) in accordance with, for example the Direct freeze thaw method (An et al., "Plant Mol. Biol. Mannual", A3: 1-19, 1988). The Agrobacterium transformed with the chimeric gene is cultivated overnight. Proliferated cells are collected by centrifugation at 5000 rpm for 5 minutes, and they are suspended in B5 suspension medium. Soybean seeds are sterilized, and they are cultivated for 3 days on B5 medium having a 1/10 concentration so that they germinate. Cotyledons are excised, and they are cultivated for 2 hours with the suspension of Agrobacterium. The cotyledons are transferred to B5 medium (containing Gamborg B5 salt (Exp. Cell. Res., 50, 151 (1968)), Gamborg B5 vitamin, 3 % sucrose, 5 μ M benzylaminopurine, 10 μ M IBA, and 100 μ M acetosyringon), and they are cultivated for 3 days under a condition at 25 °C with light irradiation ($60 \mu\text{Em}^{-2}\text{S}^{-1}$) for 23 hours. Subsequently, in order to remove Agrobacterium, the cotyledons are cultivated in B5 medium (5 μ M benzylaminopurine, 10 mg/L carbenicillin, 100 mg/L

vancomycin, and 500 mg/L cefotaxime) at 25 °C for 4 days while exchanging the medium every day. After that, the cotyledons are cultivated in B5 medium (200 mg/L kanamycin). Multishoots are formed within 1 or 2 months. They are cultivated on B5 medium (0.58 mg/L gibberellin and 50 mg/L kanamycin) to elongate the shoots. Subsequently, the shoots are transferred to B5 medium (10 µM IBA) to cause rooting. Rooted seedlings are acclimatized, and they are cultivated in a greenhouse. Thus transformants can be obtained.

A transformant plant, in which the raffinose synthase gene is introduced, can be easily confirmed by extracting DNA from the transformant, and performing Southern hybridization by using the raffinose synthase gene as a probe.

Brief Description of the Drawings

Fig. 1 shows a relationship between the reaction time and the amount of raffinose produced by the raffinose synthesis reaction.

Fig. 2 shows a photograph illustrating a result of SDS-polyacrylamide gel electrophoresis for the raffinose synthase. M indicates molecular weight markers, and S indicates a sample containing the raffinose synthase. Numerals indicate molecular weights (kDa).

Fig. 3 shows an influence of the reaction

temperature on the raffinose synthase activity.

Fig. 4 shows an influence of the reaction pH on the raffinose synthase activity.

Fig. 5 shows an influence of myo-inositol on the 5 raffinose synthase activity.

Fig. 6 shows a stable pH range of the raffinose synthase.

Fig. 7 shows relationships between synthetic primers and amino acid sequences of peptides. R 10 represents A or G, Y represents C or T, M represents A or C, K represents G or T, D represents G, A, or T, H represents A, T, or C, B represents G, T, or C, N represents G, A, T, or C, and I represents inosine.

Best Mode for Carrying Out the Invention

15 The present invention will be more specifically explained below with reference to Examples.

At first, the method for measuring the raffinose synthase activity, used to confirm active fractions during respective purification steps and investigate 20 characteristics of the enzyme in the following Examples, will be explained.

<Method for measuring the raffinose synthase activity>

The activity of the raffinose synthase was measured by quantitatively determining raffinose produced by the

raffinose synthesis reaction by using HPLC (high-performance liquid chromatography). HPLC was performed by using Sugar Analysis System DX500 (CarboPac PA1 column, pulsed amperometry detector (produced by 5 DIONEX)).

The raffinose synthesis reaction was performed by using a reaction solution prepared to have a composition having the following final concentrations. The reaction solution was added with 10 to 50 µl of a raffinose 10 synthase solution to give a volume of 100 µl, followed by performing the reaction at 32 °C for 60 minutes.

[Composition of reaction solution (final concentration)]

	2.5 mM sucrose
	5 mM galactinol
15	5 mM DTT
20	20 mM Tris-HCl buffer (pH 7.0)

After performing the reaction as described above, the reaction solution was added with ethanol in a volume four times the volume of the reaction solution to stop 20 the reaction by heating the solution at 95 °C for 30 seconds. The obtained solution was centrifuged to obtain a supernatant which was then dried up under a reduced pressure. After that, an obtained residue was dissolved in distilled water. Raffinose in the reaction 25 product was quantitatively determined by using the sugar

analysis system to estimate the raffinose synthase activity.

Example 1: Purification of Raffinose Synthase
from Cucumber

5 <1> Extraction of raffinose synthase from cucumber

Vein tissues were collected from true leaves of cucumber (cv.: SUYOU) obtained 6 to 10 weeks after planting. The leaf vein tissues were frozen with liquid nitrogen, and they were stored at -80 °C. The frozen 10 leaf vein tissues were ground by a mortar with liquid nitrogen, to which Buffer 1 (40 mM Tris-HCl buffer (pH 7.0), 5 mM DTT, 1 mM PMSF (phenylmethanesulfonyl fluoride), 1 % polyclarl AT (produced by Serva)) was added to extract proteins. An obtained extract solution 15 was filtrated with a filter such as gauze or Miracloth (produced by Calbiochem-Novobiochem). An obtained filtrate was centrifuged at 4 °C at about 30,000 x g for 60 minutes. A supernatant obtained by the centrifugation was used as a crude extract solution.

20 <2> Anion exchange chromatography (1)

The crude extract solution (about 560 ml) obtained as described above was applied to a column system comprising five connected columns for strongly basic anion exchange chromatography (HiTrap Q, produced by

Pharmacia, 1.6 cm x 2.5 cm) equilibrated with Buffer 2 (20 mM Tris-HCl buffer (pH 7.0), 5 mM DTT) to adsorb the raffinose synthase activity to the columns.

Subsequently, the columns were washed with Buffer 3 (20
5 mM Tris-HCl buffer (pH 7.0), 0.2 M NaCl, 5 mM DTT) in a
volume five times of the columns so that non-adsorbed
proteins were washed out. After that, the raffinose
synthase activity was eluted from the columns with 50 ml
of Buffer 4 (20 mM Tris-HCl buffer (pH 7.0), 0.3 M NaCl,
10 5 mM DTT).

<3> Anion exchange chromatography (2)

The eluted solution (about 75 ml) was placed in a
dialysis tube (Pormembranes MWC 0:10,000, produced by
Spectra), and it was dialyzed against 10 L of Buffer 5
15 (20 mM Tris-HCl buffer (pH 7.0), 0.05 M NaCl, 5 mM DTT)
at 4 °C overnight. The dialyzed sample was applied to a
column for weakly basic anion exchange chromatography
(DEAE-TOYOPEARL, produced by Toyo Soda, 2.2 x 20 cm)
equilibrated with Buffer 5 to adsorb the raffinose
20 synthase activity to the column. Subsequently, the
column was washed with Buffer 5 in a volume five times
the volume of the column to wash out non-adsorbed
proteins. After that, a linear concentration gradient
of 0.05 M to 0.35 M NaCl in a volume twenty times the
25 volume of the column was applied to elute the enzyme
activity so that fractionation was performed.

<4> Gel filtration chromatography

The eluted solution obtained as described above (about 160 ml) was concentrated into 6.5 ml by using a concentrator (Centriprep 10, produced by Amicon).
5 Aliquots (each 3 ml) of the concentrated solution were applied to a column for gel filtration chromatography (Superdex 200 pg, produced by Pharmacia, 2.6 cm x 60 cm). Equilibration for the column and elution from the column were performed by using Buffer 6 (20 mM Tris-HCl
10 buffer (pH 7.0), 0.1 M NaCl, 5 mM DTT, 0.02 % Tween 20). Fractions having the raffinose synthase activity were collected from fractionated fractions.

<5> Hydroxyapatite chromatography

A collected fraction (about 25 ml) having the raffinose synthase activity fractionated by the gel filtration was concentrated by using Centriprep 10, and the buffer was exchanged with Buffer 7 (0.01 M sodium phosphate buffer (pH 7.0), 5 mM DTT, 0.02 % Tween 20). An obtained concentrate solution (about 1.2 ml) was applied to a hydroxyapatite column (Bio-Scale CHT-1, produced by Bio Rad, 0.7 x 5.2) previously equilibrated with the same buffer to adsorb the raffinose synthase activity. The column was washed with the same buffer in a volume (10 ml) five times the volume of the column.
20 After that, a linear concentration gradient of 0.01 M to 0.3 M phosphate in a volume twenty times the volume of
25

the column was applied to elute the enzyme activity so that fractionation was performed.

<6> Hydroxyapatite rechromatography

An active fraction obtained in accordance with the 5 hydroxyapatite chromatography as described above was subjected to rechromatography in the same manner as described above to obtain a purified raffinose synthase fraction (about 2 ml).

The amount of protein contained in the active 10 fraction was about 200 µg. The total activity was 5700 nmol/hour, and the specific activity per protein was 28 µmol/hour/mg. The active fraction contained only a protein which exhibited a single band corresponding to a molecular weight of 90 kDa to 100 kDa on electrophoresis 15 as described later on. The specific activity of the obtained purified enzyme sample was about 2000 times that of the crude extract solution. The recovery was 12 % with respect to the amount of the enzyme obtained after the strongly basic anion exchange chromatography 20 based on the use of HiTrap Q. Results of the purification are summarized in Table 1.

Table 1

		<u>Total</u> <u>protein</u>	<u>Total</u> <u>activity</u>	<u>Specific</u> <u>activity</u>	<u>Yield</u> <u>%</u>
		<u>mg</u>	<u>nmol/h</u>	<u>nmol/h/mg</u>	
5	Crude extract	1915	20700	11	-
	HiTrap Q	1092	48800	45	100
	DEAE-TOYOPEARL	540	33000	61	68
	Superdex 200 pg	1.79	26500	14800	54
	Apatite (1)*	0.51	12600	24700	26
10	Apatite (2)*	0.20	5700	28500	12

Apatite (1)*: Hydroxyapatite chromatography (1)

Apatite (2)*: Hydroxyapatite chromatography (2)

Example 2: Investigation on Characteristics

of Raffinose Synthase

15 Characteristics of the purified raffinose synthase obtained in Example 1 were investigated.

<1> Molecular weight measurement

(1) Gel filtration chromatography

20 An aliquot (10 µl) of the purified raffinose synthase was dispensed. This sample and a molecular weight marker (Molecular Weight Marker Kit for Gel Filtration, produced by Pharmacia) were applied to a gel

filtration chromatography column (Superdex 200 pg, produced by Pharmacia). Equilibration of the column and elution from the column were performed by using Buffer 6 (20 mM Tris-HCl buffer (pH 7.0), 0.1 M NaCl, 5 mM DTT, 5 0.02 % Tween 20). As a result, the molecular weight of the raffinose synthase was estimated to be about 75 kDa to 95 kDa.

(2) Polyacrylamide gel electrophoresis (Native PAGE)

An aliquot (10 μ l) of the purified raffinose 10 synthase was dispensed, to which the same volume of a sample buffer (0.0625 M Tris-HCl (pH 6.8), 15 % glycerol, 0.001 % BPB) was added to prepare an electrophoresis sample. The sample (10 μ l) was applied 15 to 10 % polyacrylamide gel (produced by Daiichi Chemical, Multigel 10), and electrophoresed at 40 mA for about 60 minutes with 0.025 M Tris - 0.192 M glycine buffer (pH 8.4). After the electrophoresis, the gel was stained with Silver Stain Kit (produced by nacalai tesque). As a result, the molecular weight was 20 estimated to be about 90 kDa to 100 kDa.

(3) SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

An aliquot (10 μ l) of the purified raffinose 25 synthase was dispensed, to which the same volume of a sample buffer (0.0625 M Tris-HCl (pH 6.8), 2 % SDS, 10 % glycerol, 5 % mercaptoethanol, 0.001 % BPB) was added, followed by heating in a boiling water bath for 1 minute to prepare an electrophoresis sample. The sample (10

μl) was applied to 10 to 20 % gradient polyacrylamide gel (produced by Daiichi Chemical), and electrophoresed at 40 mA for about 70 minutes with 0.025 M Tris - 0.192 M glycine buffer (pH 8.4) containing 0.1 % SDS. After 5 the electrophoresis, the gel was stained with Silver Stain Kit (produced by nacalai tesque). A result is shown in Fig. 2. As a result, the molecular weight was estimated to be about 90 kDa to 100 kDa.

<2> Optimum reaction temperature

10 The raffinose synthase activity was measured under various temperature conditions (28 °C, 32 °C, 36 °C, 40 °C, 44 °C, 48 °C, and 52 °C) in accordance with the method for measuring the raffinose synthase activity described above. The enzyme solution was added to the 15 respective reaction solutions in an amount of 2 μl. Fig. 3 shows relative activities at the respective temperatures assuming that the enzyme activity at 32 °C was 100. As a result, the raffinose synthase exhibited the activity in a range of about 25 to 42 °C, and the 20 optimum reaction temperature was about 35 to 40 °C.

<3> Optimum reaction pH

The raffinose synthase activity was measured under various pH conditions (pH 4 to 11) in accordance with the method for measuring the raffinose synthase activity 25 described above. The respective reactions were

performed by using 50 mM citrate buffer (pH 4 to 6), 50 mM potassium phosphate buffer (pH 5.5 to 7.5), 50 mM Bis-Tris buffer (pH 6 to 7), 20 mM Tris-HCl buffer (pH 7 to 8.5), and 50 mM glycine-NaOH buffer (pH 9 to 11).
5 The enzyme solution was added to the respective reaction solutions in an amount of 2 μ l. A result is shown in Fig. 4.

As a result, the raffinose synthase exhibited the activity in a range of pH 5 to 10, and the optimum 10 reaction pH was about 6 to 8, provided that the activity varied depending on the type of the buffer used for the measurement.

<4> Investigation on inhibitors and metal ions
Various enzyme inhibitors or metal ions were added 15 to the reaction solution of the purified raffinose synthase to give a final concentration of 1 mM respectively, and the raffinose synthase activity was measured in the same manner as described above. Table 2 shows remaining activities with respect to the enzyme 20 activity obtained when neither inhibitor nor metal ion was added. Iodoacetamide and N-ethylmaleimide clearly inhibited the enzyme activity. The inhibiting effect was scarcely observed for CaCl_2 , CuCl_2 , and MgCl_2 . However, MnCl_2 , ZnCl_2 , and NiCl_2 exhibited the inhibiting 25 effect.

Table 2

	<u>Inhibitor or metal ion</u>	<u>Remaining activity (%)</u>
	No addition	100
	Iodoacetoamide	0
5	N-ethylmaleimide	40
	CaCl ₂	115
	CuCl ₂	101
	MgCl ₂	96
	MnCl ₂	32
10	ZnCl ₂	42
	NiCl ₂	68

<5> Inhibition by myo-inositol

Investigation was made for inhibition by myo-inositol as the reaction product of the raffinose synthesis reaction. The reaction solution was added with myo-inositol at various concentrations, and the raffinose synthase activity was measured. A result is shown in Fig. 5. The enzyme activity was inhibited as the concentration of added myo-inositol was increased.

20 <6> Stable pH

The raffinose synthase fraction obtained by the anion exchange chromatography (2) described above was incubated for 4 hours at 4 °C in 50 mM Bis-Tris-HCl buffer (pH 5 to 8.0, containing 0.5 mM DTT) or 20 mM

Tris-HCl buffer (pH 7 to 8.0, containing 0.5 mM DTT), and then the raffinose synthase activity was measured. Fig. 6 shows the enzyme activity versus pH of the buffer used for the incubation. The raffinose synthase 5 activity was confirmed after the incubation under any of the incubation conditions. Especially, the enzyme was stable in a range of pH 5 to 7.5.

<7> Stable temperature

The raffinose synthase fraction obtained by the 10 anion exchange chromatography (2) described above was incubated in 20 mM Tris-HCl buffer (pH 7, containing 0.5 mM DTT) for 60 minutes at 28 °C, 32 °C, 37 °C, or 40 °C, and then the raffinose synthase activity was measured. As a result, the enzyme of the present invention was 15 stable, exhibiting, in the range of 28 °C to 40 °C, activities of 80 % to 100 % of that obtained by a control for which the incubation treatment was not performed for comparison.

<8> Analysis of amino acid sequence

20 The cysteine residue of the purified raffinose synthase was subjected to reducing pyridylethylation, and the reaction mixture was desalted. An obtained sample was digested at 37 °C for 12 hours with lysylendopeptidase (Achromobacter protease 1, produced 25 by Wako Pure Chemical Industries) to form peptide

fragments. An obtained peptide mixture was applied to reverse phase HPLC (column: Waters μBondasphere (φ2.1 x 150 mm, C₁₈, 300 Å, produced by Waters (Millipore))) to separate and obtain the respective peptide fragments.

5 0.1 % TFA (trifluoroacetic acid) was used as a solvent, and elution was performed with a concentration gradient of acetonitrile. Amino acid sequences of three fragments selected from the obtained peptide fragments were determined by using a protein sequencer. The

10 determined amino acid sequences of the respective peptides are shown in SEQ ID NOs: 1 to 3 in Sequence Listing. These peptides will be thereafter referred to as Peptides 1, 2, and 3 respectively in this order.

15 Example 3: Preparation of DNA
 Coding for Raffinose Synthase

<1> Isolation of partial fragment of cDNA of raffinose synthase by means of PCR method

Major veins of cucumber (22 g) were ground by a mortar with liquid nitrogen. The ground material was

20 added to a mixture of an extraction buffer (100 mM lithium chloride, 100 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 1 % SDS) and an equal amount of phenol previously heated to 80 °C, followed by agitation. After that, a mixture of phenol and an equal amount of chloroform:

25 isoamyl alcohol (24:1) was added thereto, followed by

agitation again. An obtained mixture solution was centrifuged at 4 °C at 9250 x g for 15 minutes to collect a supernatant. The supernatant was repeatedly subjected to the treatment with phenol and the treatment with chloroform: isoamyl alcohol to obtain a supernatant after centrifugation. The supernatant was added with an equal amount of 4 M lithium chloride, followed by being stationarily left to stand at -70 °C for 1 hour.

After thawing at room temperature, the sample was treated and centrifuged at 4 °C at 9250 x g for 30 minutes to obtain a precipitate. The precipitate was washed with 2 M lithium chloride once and with 80 % ethanol once. After drying, the precipitate was dissolved in 2 ml of a diethylpyrocarbonate-treated solution to give a sample of purified total RNA. The obtained total RNA was 2.38 mg.

The all amount of the total RNA was applied to poly(A)⁺RNA purification kit (produced by STRATAGENE CLONING SYSTEMS) based on the use of an oligo(dT) cellulose column so that poly(A)⁺RNA molecules were purified to obtain 42.5 µg of poly(A)⁺RNA.

Single strand cDNA's were synthesized from poly(A)⁺RNA obtained as described above, by using reverse transcriptase Super Script II (produced by GIBCO BRL). In order to isolate raffinose synthase cDNA from an obtained cDNA mixture, amplification was performed in accordance with the PCR method. In order to be used as

primers in PCR, single strand oligonucleotides (SEQ ID
NOS: 6 to 22) shown in Fig. 7 were synthesized on the
basis of the amino acid sequences of the peptide
fragments of the raffinose synthase originating from
5 cucumber, determined in Example 2. In the sequences of
the respective primers, R represents A or G, Y
represents C or T, M represents A or C, K represents G
or T, D represents G, A, or T, H represents A, T, or C,
B represents G, T, or C, N represents G, A, T, or C, and
10 I represents inosine (base: hypoxanthine) respectively.

A DNA fragment of about 540 base pairs was
amplified when the primers were combined and used such
that the 5'-side primer was A (A1 (SEQ ID NO: 6), A2
(SEQ ID NO: 7), A3 (SEQ ID NO: 8), A4 (SEQ ID NO: 9))
15 and the 3'-side primer was D' (D'1 (SEQ ID NO: 21), D'2
(SEQ ID NO: 22)), or the 5'-side primer was C2 (SEQ ID
NO: 14) and the 3'-side primer was B'1 (SEQ ID NO: 18)
or B'2 (SEQ ID NO: 19). The fragment was cloned into a
plasmid pCRII by using TA cloning kit (produced by
20 INVITROGEN BV) to analyze its nucleotide sequence. As a
result, a nucleotide sequence coding for the amino acid
sequences of Peptides 1, 2 was found inwardly between
the primer sequences at both terminals. Accordingly, it
was found that the amplified fragment is a DNA fragment
25 originating from the raffinose synthase gene.

In order to specify the position of the cloned PCR-
amplified DNA fragment on the raffinose synthase gene,

3'-RACE was performed by using RACE kit (3' Ampifinder RACE Kit, produced by CLONTACH).

PCR was performed by using the cDNA mixture as a template, C (C1 (SEQ ID NO: 13), C2 (SEQ ID NO: 14)) as 5 a 5'-side primer, and a primer having oligo(dT) and an anchor sequence as a 3'-side primer. Further, PCR was performed by using an amplified fragment thus obtained as a template, D (D1 (SEQ ID NO: 15), D2 (SEQ ID NO: 16)) located inwardly from C as a 5'-side primer, and an 10 oligo(dT)-anchor primer as a 3'-side primer. As a result, a DNA fragment of about 2400 base pairs was amplified only when PCR was performed by using, as the template, DNA amplified with C1 (SEQ ID NO: 13) or C2 (SEQ ID NO: 14) and the oligo(dT)-anchor primer, and 15 using D2 (SEQ ID NO: 16) and the oligo(dT)-anchor primer. Further, PCR was performed by using C (C1 (SEQ ID NO: 13), C2 (SEQ ID NO: 14)) as the 5'-side primer and the oligo(dT)-anchor primer as the 3'-side primer, and then PCR was performed by using the amplified 20 fragment thus obtained as a template, E (SEQ ID NO: 17) as a 5'-side primer, and the oligo(dT)-anchor primer as a 3'-side primer. As a result, a DNA fragment of about 300 base pairs was amplified in any case.

Similarly, PCR was performed by using A (A1 (SEQ ID 25 NO: 6), A2 (SEQ ID NO: 7), A3 (SEQ ID NO: 8), or A4 (SEQ ID NO: 9)) as a 5'-side primer, and the primer having oligo(dT) and the anchor sequence as a 3'-side primer.

Further, PCR was performed by using an amplified fragment thus obtained as a template, and using B (B1 (SEQ ID NO: 10), B2 (SEQ ID NO: 11), or B3 (SEQ ID NO: 12)) located inwardly from A as a 5'-side primer, and 5 the same oligo(dT)-anchor primer as a 3'-side primer. As a result, a DNA fragment of about 2000 base pairs was obtained when the B2 primer was used even when any of the A primers was used. Thus the DNA fragment amplified by using the A2 and B2 primers was cloned. As a result 10 of nucleotide sequence analysis, the DNA fragment included the nucleotide sequence coding for the amino acid sequence of Peptide fragment 1 used to prepare the 5'-side primer. The DNA fragment also included, on the 3'-side, the poly(A) sequence and the nucleotide 15 sequence corresponding to Peptide fragment 3 at a position located upstream therefrom.

In view of the result of PCR described above, it was found that Peptide fragments of the raffinose synthase are arranged from the N-terminal side in an 20 order of 2, 1, 3, and the DNA fragment of about 540 base pairs previously obtained by PCR was a portion located near to the 5'-terminal on the raffinose synthase gene. In order to screen a cDNA clone containing the entire 25 length of the raffinose synthase gene, it is desirable that DNA to be used as a probe can detect a portion near to the 5'-terminal side. Accordingly, the obtained DNA fragment was used as a probe to perform screening for a

CDNA library.

<2> Cloning of entire length of coding region of raffinose synthase cDNA

At first, a cDNA library was prepared as follows.

5 Double strand cDNA's were synthesized from poly(A)⁺RNA (3.8 µg) obtained in the foregoing item <1> by using Time Saver cDNA synthesis kit (produced by Pharmacia Biotech). Obtained cDNA's were incorporated into EcoRI restriction enzyme cleavage site of λ phage vector,
10 λMOSSllox (produced by Amersham) respectively, which were then incorporated into the phage protein by using GigapackII Gold packaging kit (produced by STRATAGENE CLONING SYSTEMS). Thus the cucumber cDNA library was prepared. This library had a titer of 1.46×10^7 pfu/µg
15 vector.

Host cells of Escherichia coli ER1647 were infected with the phages contained in the cucumber cDNA library in an amount corresponding to 1.4×10^5 pfu, and then the cells were spread over 14 agar plates each having a diameter of 90 mm to give 1.0×10^4 pfu per one plate.
20 The cells were cultivated at 37 °C for about 6.5 hours. After that, phage plaques formed on the plates were transferred to nylon membranes (Hybond-N+, produced by Amersham).

25 Next, the nylon membranes were treated with alkali to denature transferred DNA, followed by neutralization

and washing. After that, the nylon membranes were treated at 80 °C for 2 hours to fix DNA on the membranes.

Positive clones were screened on the obtained nylon membrane by using the DNA fragment of about 540 base pairs obtained in the foregoing item <1> as a probe. The DNA fragment of about 540 base pairs was digested with restriction enzyme EcoRI, followed by electrophoresis to excise and purify only the insert of about 540 base pairs. The insert was labeled with fluorescein by using DNA labeling and detection system (Gene Images labeling and detection system, produced by Amersham) to be used as the probe. The nylon membranes were subjected to prehybridization at 60 °C for 30 minutes, and then the labeled probe was added to perform hybridization at 60 °C for 16 hours. An antibody (alkaline phosphatase-labeled anti-fluorescein antibody) for detecting the labeled DNA was used after being diluted 50000 times. In this screening process, candidate strains for positive clones were obtained. The obtained candidate strains were further subjected to repeated screening twice in the same manner as described above to obtain a purified positive clone.

Escherichia coli BM25.8 was infected with the positive clone, and it was cultivated on a selection medium containing carbenicillin. A plasmid vector λMOSSlox-CRS containing cDNA was excised therefrom. The

inserted cDNA of the plasmid had a length of about 2.5 kb. Escherichia coli JM109 was transformed with the plasmid. Plasmid DNA was prepared from a transformant, which was used as a sample for analyzing the nucleotide sequence.

The nucleotide sequence of the inserted cDNA was analyzed by using Taq DyeDeoxy Terminator Cycle Sequencing Kit (produced by Perkin-Elmer) in accordance with the conventionally known method.

As a result, a nucleotide sequence comprising 2352 base pairs as shown in SEQ ID NO: 4 in Sequence Listing was revealed. The sequence included a portion coincident with the nucleotide sequence of the DNA probe used by the present inventors. An amino acid sequence translated from the nucleotide sequence is shown in SEQ ID NOs: 4 and 5. The amino acid sequence included portions coincident with Peptide 1 (amino acid numbers of 215 to 244 in SEQ ID NO: 5), Peptide 2 (amino acid numbers of 61 to 79 in SEQ ID NO: 5), and Peptide 3 (amino acid numbers of 756 to 769 in SEQ ID NO: 5) of the raffinose synthase originating from cucumber obtained by the present inventors. Thus it was confirmed that the amino acid sequence codes for the raffinose synthase.

The transformant, designated as AJ13263, of Escherichia coli JM109, which harbors the plasmid pMossloxCRS containing DNA coding for the raffinose

synthase obtained as described above, has been internationally deposited on the basis of the Budapest Treaty since November 19, 1996 in National Institute of Bioscience and Human Technology of Agency of Industrial Science and Technology of Ministry of International Trade and Industry (postal code: 305, 1-3 Higashi-Ichchome, Tsukuba-shi, Ibaraki-ken, Japan), and awarded a deposition number of FERM BP-5748.

Example 4: Chimeric gene and Transformed Plant
Containing DNA Coding for Raffinose Synthase

<1> Construction of plasmid containing chimeric gene
The DNA fragment coding for the raffinose synthase was introduced into Arabidopsis thaliana by using LBA4404 as Agrobacterium and pBI121 (produced by CLONTECH) as a binary vector. pBI121 is a plasmid originating from pBIN19, which comprises nopaline synthase gene promoter connected to neomycin phosphotransferase structural gene (NPTII), nopaline synthase gene terminator (Nos-ter), CaMV 35S promoter, GUS (β -glucuronidase) gene, and Nos-ter, and which has sequences for enabling transposition to plant, on both sides thereof. A SmaI site is located downstream from CaMV 35S promoter. An insert inserted into this site is expressed under the regulation of the promoter.

A fragment of the raffinose synthase gene obtained

in Example 3 was inserted into the binary vector pBI121. The raffinose synthase gene was digested with DraI to prepare, by means of agarose gel electrophoresis, a DNA fragment containing 30th to 1342th nucleotides in SEQ ID 5 NO: 4 in Sequence Listing. This fragment was ligated into the SmaI site of pBI121. Escherichia coli HB101 was transformed with the ligation reaction solution to obtain transformant strains from which recombinant plasmids were prepared. Two recombinant plasmids, in 10 which the raffinose synthase DNA fragment was reversely connected to CaMV 35S promoter (antisense), and the raffinose synthase DNA fragment was connected to CaMV 35S promoter in the positive direction (sense), were selected from the obtained recombinant plasmids. The 15 two recombinant plasmids were designated as pBIRS1 and pBIRS9 respectively.

Each of the plasmids obtained as described above was introduced into Agrobacterium LBA4404 by means of triparental mating.

20 Arabidopsis thaliana was transformed as follows. Seeds of Arabidopsis thaliana was subjected to a treatment for water absorption. After that, they were sterilized by treating them with 80 % ethanol containing 1 % Tween 20 for 5 minutes, and treating them with 10 % 25 sodium hypochlorite solution also containing 1 % Tween 20 for 10 minutes, followed by washing five times with sterilized water. The seeds were suspended in 1 % low

melting point agarose, and they were spread over an MS medium (MS basic medium (Murashige and Skoog, Physiologia Plantrum, 15, 473-497 (1962)), B5 vitamin, 10 g/L sucrose, 0.5 g/L MES, pH 5.8). The seeds were
5 cultivated at 22 °C for 1 week in a culture room to give a cycle comprising light irradiation for 16 hours and darkness for 8 hours. Plants with unfolded seed leaves were subjected to setting with rock wool. Cultivation was continued under the same condition. After about 3
10 weeks, decapitation was performed when the plants caused bolting to have heights of stems of several cm's. The plants were allowed to grow until a state in which first flowers bloom on elongated branches 1 week after the decapitation.

15 Agrobacterium harboring the introduced recombinant plasmid containing the raffinose synthase gene was precultivated in 2 ml of LB medium. An obtained culture was inoculated into LB medium containing 50 mg/L kanamycin and 25 mg/L streptomycin, followed by
20 cultivation at 28 °C for about 1 day. Bacterial cells were collected at room temperature, and they were suspended in a suspension medium for infiltration (1/2 MS salt, 1/2 Gamborg B5 vitamin, 5 % sucrose, 0.5 g/L MES, pH 5.7 (KOH), to which, immediately before the use,
25 benzylaminopurine was added to give a final concentration of 0.044 µM, or Silwet L77 was added in an amount of 200 µl per 1 L (final concentration: 0.02 %))

so that OD₆₀₀ of an obtained bacterial suspension was 0.8.

Flowers in bloom and fructification were removed from the plants to be subjected to infiltration. The 5 rock wool was inverted upside down, and flowers which were not in fructification were immersed in the suspension of Agrobacterium, followed by being placed in a desiccator so that the pressure was reduced to be 40 mmHG for 15 minutes. Seeds were harvested after 2 to 4 10 weeks. The harvested seeds were stored in a desiccator.

Next, transformants were selected on a selection medium. The seeds were sterilized in the same manner as described above, and they were cultivated on a selection medium (MS salt, Gamborg B5 vitamin, 1 % sucrose, 0.5 15 g/L MES, pH 5.8, 0.8 % agar, to which antibiotics for selection, i.e., carbenicillin (final concentration: 100 mg/L) and kanamycin (final concentration: 50 mg/L) were added after autoclaving)) at 22 °C to select resistant plants. The resistant plants were transferred to a 20 fresh medium, and they were allowed to grow until true leaves unfolded. Seeds were harvested from the obtained plants. Selection was repeated in the same manner as described above, and thus T3 seeds were obtained. The T3 seeds were measured for the raffinose content in 25 accordance with the method described above. Results are shown in Table 4.

Table 4

<u>Plant</u>	<u>Raffinose content (mg/g)</u>
Wild type	0.2
Transformant (pBIRS1)	0.0
5 Transformant (pBIRS9)	0.0

Industrial Applicability

The present invention provides the purified raffinose synthase, the raffinose synthase gene, the chimeric gene comprising the raffinose synthase gene and
10 the regulatory region expressible in plants, and the plant introduced with the chimeric gene.

Raffinose can be efficiently synthesized from sucrose and galactinol by using the raffinose synthase of the present invention. The content of the raffinose
15 family oligosaccharides in plants can be changed by utilizing the raffinose synthase gene or the chimeric gene of the present invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: OSUMI Chieko
NOZAKI Jinshi
KIDA Takao

(ii) TITLE OF INVENTION: RAFFINOSE SYNTHASE GENE, METHOD FOR
PRODUCING RAFFINOSE, AND TRANSGENIC PLANT

(iii) NUMBER OF SEQUENCES: 22

(iv) CORRESPONDENCE ADDRESS:

- (A) ADDRESSEE:
- (B) STREET:
- (C) CITY:
- (E) COUNTRY:
- (F) ZIP:

(v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(vi) ATTORNEY/AGENT INFORMATION:

- (A) NAME:
- (B) REGISTRATION NUMBER:

(vii) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE:
- (B) TELEFAX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Phe Gly Trp Cys Thr Trp Asp Ala Phe Tyr Leu Thr Val His Pro Gln
1 5 10 15

Gly Val Ile Glu Gly Val Arg His Leu Val Asp Gly Gly Cys
20 25 30

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 amino acids
- (B) TYPE: amino acid

- (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (v) FRAGMENT TYPE: internal
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Pro Val Ser Val Gly Cys Phe Val Gly Phe Asp Ala Ser Glu Pro Asp
1 5 10 15
Ser Arg His

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 14 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (v) FRAGMENT TYPE: internal

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Tyr Asp Gln Asp Gln Met Val Val Val Gln Val Pro Trp Pro
1 5 10

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 2517 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA to mRNA
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: cucumber (*Cucumis sativas*)
- (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 56..2407
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AAAAAAACAAC CCTTCTTTA GTTTTTGGG TTTGTTCTT CTTTCTTCT CACAA ATG	58
Met	
1	
GCT CCT AGT TTT AAA AAT GGT GGC TCC AAC GTA GTT TCA TTT GAT GGC	106
Ala Pro Ser Phe Lys Asn Gly Gly Ser Asn Val Val Ser Phe Asp Gly	
5 10 15	
TTA AAT GAC ATG TCG TCA CCG TTT GCA ATC GAC GGA TCG GAT TTC ACT	154
Leu Asn Asp Met Ser Ser Pro Phe Ala Ile Asp Gly Ser Asp Phe Thr	
20 25 30	
GTG AAC GGT CAT TCG TTT CTG TCC GAT GTT CCT GAG AAC ATT GTT GCT	202
Val Asn Gly His Ser Phe Leu Ser Asp Val Pro Glu Asn Ile Val Ala	
35 40 45	
TCT CCT TCT CCG TAC ACT TCG ATA GAC AAG TCC CCG GTT TCG GTT GGT	250

Ser Pro Ser Pro Tyr Thr Ser Ile Asp Lys Ser Pro Val Ser Val Gly				
50	55	60	65	
TGC TTT GTT GGA TTC GAC GCG TCG GAA CCT GAT AGC CGA CAT GTT GTT				298
Cys Phe Val Gly Phe Asp Ala Ser Glu Pro Asp Ser Arg His Val Val				
70	75	80		
TCG ATT GGG AAG CTG AAG GAT ATT CCG TTT ATG AGT ATT TTC AGG TTT				346
Ser Ile Gly Lys Leu Lys Asp Ile Arg Phe Met Ser Ile Phe Arg Phe				
85	90	95		
AAG GTT TGG TGG ACT ACA CAC TGG GTT GGT CGA AAT GGT GGG GAT CTT				394
Lys Val Trp Trp Thr Thr His Trp Val Gly Arg Asn Gly Gly Asp Leu				
100	105	110		
GAA TCG GAG ACT CAG ATT GTG ATC CTT GAG AAG TCA GAT TCT GGT CGA				442
Glu Ser Glu Thr Gln Ile Val Ile Leu Glu Lys Ser Asp Ser Gly Arg				
115	120	125		
CCG TAT GTT TTC CTT CTT CCG ATC GTT GAG GGA CCG TTC CGA ACC TCG				490
Pro Tyr Val Phe Leu Leu Pro Ile Val Glu Gly Pro Phe Arg Thr Ser				
130	135	140	145	
ATT CAG CCT GGG GAT GAT GAC TTT GTC GAT GTT TGT GTC GAG AGT GGT				538
Ile Gln Pro Gly Asp Asp Asp Phe Val Asp Val Cys Val Glu Ser Gly				
150	155	160		
TCG TCG AAA GTT GTT GAT GCA TCG TTC CGA AGT ATG TTG TAT CTT CAT				586
Ser Ser Lys Val Val Asp Ala Ser Phe Arg Ser Met Leu Tyr Leu His				
165	170	175		
GCT GGT GAT GAT CCG TTT GCA CTT GTT AAA GAG GCG ATG AAG ATC GTG				634
Ala Gly Asp Asp Pro Phe Ala Leu Val Lys Glu Ala Met Lys Ile Val				
180	185	190		
AGG ACC CAT CTT GGA ACT TTT CGC TTG TTG GAG GAG AAG ACT CCA CCA				682
Arg Thr His Leu Gly Thr Phe Arg Leu Leu Glu Glu Lys Thr Pro Pro				
195	200	205		
GGT ATC GTG GAC AAA TTC GGT TGG TGC ACG TGG GAC GCG TTT TAC CTA				730
Gly Ile Val Asp Lys Phe Gly Trp Cys Thr Trp Asp Ala Phe Tyr Leu				
210	215	220	225	
ACG GTT CAT CCA CAG GGC GTA ATA GAA GGC GTG AGG CAT CTC GTC GAC				778
Thr Val His Pro Gln Gly Val Ile Glu Gly Val Arg His Leu Val Asp				
230	235	240		
GGC GGT TGT CCT CCC GGT TTA GTC CTA ATC GAC GAT GGT TGG CAA TCC				826
Gly Gly Cys Pro Pro Gly Leu Val Leu Ile Asp Asp Gly Trp Gln Ser				
245	250	255		
ATC GGA CAC GAT TCG GAT CCC ATC ACC AAA GAA GGA ATG AAC CAA ACC				874
Ile Gly His Asp Ser Asp Pro Ile Thr Lys Glu Gly Met Asn Gln Thr				
260	265	270		
GTC GCC GGC GAG CAA ATG CCC TGC CGT CTT TTG AAA TTC CAA GAG AAT				922
Val Ala Gly Glu Gln Met Pro Cys Arg Leu Leu Lys Phe Gln Glu Asn				
275	280	285		
TAC AAA TTC CGT GAC TAC GTC AAT CCC AAG GCC ACC GGC CCC CGA GCC				970

Tyr Lys Phe Arg Asp Tyr Val Asn Pro Lys Ala Thr Gly Pro Arg Ala			
290	295	300	305
GGC CAG AAG GGG ATG AAG GCG TTT ATA GAT GAA CTC AAA GGA GAG TTT			1018
Gly Gln Lys Gly Met Lys Ala Phe Ile Asp Glu Leu Lys Gly Glu Phe			
310	315	320	
AAG ACT GTG GAG CAT GTT TAT GTT TGG CAT GCT TTG TGT GGA TAT TGG			1066
Lys Thr Val Glu His Val Tyr Val Trp His Ala Leu Cys Gly Tyr Trp			
325	330	335	
GGT GGC CTT CGC CCG CAG GTG CCT GGC TTG CCT GAG GCA CGT GTG ATT			1114
Gly Gly Leu Arg Pro Gln Val Pro Gly Leu Pro Glu Ala Arg Val Ile			
340	345	350	
CAG CCA GTG CTT TCA CCA GGG CTG CAG ATG ACG ATG GAG GAT TTG GCG			1162
Gln Pro Val Leu Ser Pro Gly Leu Gln Met Thr Met Glu Asp Leu Ala			
355	360	365	
GTG GAT AAG ATT GTT CTT CAT AAG GTC GGG CTG GTC CCG CCG GAG AAG			1210
Val Asp Lys Ile Val Leu His Lys Val Gly Leu Val Pro Pro Glu Lys			
370	375	380	385
GCT GAG GAG ATG TAC GAA GGA CTT CAT GCT CAT TTG GAA AAA GTT GGG			1258
Ala Glu Glu Met Tyr Glu Gly Leu His Ala His Leu Glu Lys Val Gly			
390	395	400	
ATC GAC GGT GTT AAG ATT GAC GTT ATC CAC CTA TTG GAG ATG TTG TGT			1306
Ile Asp Gly Val Ile Asp Val Ile His Leu Leu Glu Met Leu Cys			
405	410	415	
GAA GAC TAT GGA GGG AGA GTG GAT TTG GCA AAG GCA TAT TAC AAA GCA			1354
Glu Asp Tyr Gly Gly Arg Val Asp Leu Ala Lys Ala Tyr Tyr Lys Ala			
420	425	430	
ATG ACC AAA TCA ATA AAT AAA CAT TTT AAA GGA AAT GGA GTC ATT GCA			1402
Met Thr Lys Ser Ile Asn Lys His Phe Lys Gly Asn Gly Val Ile Ala			
435	440	445	
AGT ATG GAA CAT TGT AAC GAC TTC ATG TTC CTT GGC ACG GAA GCT ATC			1450
Ser Met Glu His Cys Asn Asp Phe Met Phe Leu Gly Thr Glu Ala Ile			
450	455	460	465
TCT CTT GGT CGT GTT GGT GAT GAC TTT TGG TGC ACG GAC CCC TCT GGT			1498
Ser Leu Gly Arg Val Gly Asp Asp Phe Trp Cys Thr Asp Pro Ser Gly			
470	475	480	
GAT CCA AAC GGT ACG TTT TGG CTC CAA GGA TGT CAC ATG GTT CAT TGT			1546
Asp Pro Asn Gly Thr Phe Trp Leu Gln Gly Cys His Met Val His Cys			
485	490	495	
GCC AAC GAC AGC TTG TGG ATG GGG AAC TTC ATC CAC CCT GAC TGG GAT			1594
Ala Asn Asp Ser Leu Trp Met Gly Asn Phe Ile His Pro Asp Trp Asp			
500	505	510	
ATG TTC CAA TCC ACC CAC CCT TGT GCC GCC TTC CAT GCT GCC TCT CGA			1642
Met Phe Gln Ser Thr His Pro Cys Ala Ala Phe His Ala Ala Ser Arg			
515	520	525	
GCC ATC TCT GGT GGC CCG ATC TAT GTT AGT GAT TCT GTG GGA AAG CAT			1690

Ala Ile Ser Gly Gly Pro Ile Tyr Val Ser Asp Ser Val Gly Lys His
530 535 540 545 1738
AAC TTT GAT CTT CTG AAA AAA CTA GTG CTT CCT GAT GGA TCG ATC CTT
Asn Phe Asp Leu Leu Lys Lys Leu Val Leu Pro Asp Gly Ser Ile Leu
550 555 560
CGA AGT GAG TAC TAT GCA CTC CCG ACT CGC GAT TGT TTG TTT GAA GAC 1786
Arg Ser Glu Tyr Tyr Ala Leu Pro Thr Arg Asp Cys Leu Phe Glu Asp
565 570 575
CCT TTG CAT AAT GGA GAA ACT ATG CTT AAG ATT TGG AAT CTC AAC AAG 1834
Pro Leu His Asn Gly Glu Thr Met Leu Lys Ile Trp Asn Leu Asn Lys
580 585 590
TTC ACT GGA GTG ATT GGT GCA TTC AAC TGC CAA GGA GGA GGA TGG TGT 1882
Phe Thr Gly Val Ile Gly Ala Phe Asn Cys Gln Gly Gly Trp Cys
595 600 605
CGT GAG ACA CGC CGC AAC CAA TGC TTT TCA CAA TAC TCA AAA CGA GTG 1930
Arg Glu Thr Arg Arg Asn Gln Cys Phe Ser Gln Tyr Ser Lys Arg Val
610 615 620 625
ACA TCC AAA ACT AAC CCA AAA GAC ATA GAA TGG CAC AGT GGA GAA AAC 1978
Thr Ser Lys Thr Asn Pro Lys Asp Ile Glu Trp His Ser Gly Glu Asn
630 635 640
CCT ATC TCT ATT GAA GGC GTT AAA ACC TTT GCG CTT TAC CTC TAT CAA 2026
Pro Ile Ser Ile Glu Gly Val Lys Thr Phe Ala Leu Tyr Leu Tyr Gln
645 650 655
GCC AAA AAA CTT ATC CTC TCC AAG CCC TCT CAA GAT CTT GAC ATA GCT 2074
Ala Lys Lys Leu Ile Leu Ser Lys Pro Ser Gln Asp Leu Asp Ile Ala
660 665 670
CTT GAC CCA TTC GAA TTC GAG CTC ATC ACT GTT TCA CCA GTG ACC AAA 2122
Leu Asp Pro Phe Glu Phe Glu Leu Ile Thr Val Ser Pro Val Thr Lys
675 680 685
CTC ATC CAA ACT TCT CTA CAC TTT GCC CCA ATT GGG CTG GTG AAC ATG 2170
Leu Ile Gln Thr Ser Leu His Phe Ala Pro Ile Gly Leu Val Asn Met
690 695 700 705
CTT AAC ACT AGT GGA GCC ATC CAA TCT GTG GAC TAT GAC GAT GAC CTA 2218
Leu Asn Thr Ser Gly Ala Ile Gln Ser Val Asp Tyr Asp Asp Asp Leu
710 715 720
AGC TCA GTC GAG ATT GGT GTC AAA GGG TGT GGT GAG ATG CGA GTA TTT 2266
Ser Ser Val Glu Ile Gly Val Lys Gly Cys Gly Glu Met Arg Val Phe
725 730 735
GCA TCG AAA AAA CCA AGG GCT TGT CGT ATT GAT GGG GAG GAT GTT GGG 2314
Ala Ser Lys Lys Pro Arg Ala Cys Arg Ile Asp Gly Glu Asp Val Gly
740 745 750
TTC AAG TAT GAT CAG GAC CAA ATG GTG GTG GTT CAA GTG CCA TGG CCA 2362
Phe Lys Tyr Asp Gln Asp Gln Met Val Val Val Gln Val Pro Trp Pro
755 760 765
ATT GAT TCT TCA TCG GGT GGC ATT TCG GTT ATC GAG TAC TTG TTT 2407

Ile Asp Ser Ser Ser Gly Gly Ile Ser Val Ile Glu Tyr Leu Phe
770 775 780
TAATTTTAT TTATGTAAGC TCAATGATTG TTGTTGTTGT CGCTGTTGTT GCTATCAATG 2467
TATTCTCTC CAAAAGAAAA TTATGTGAA TTTGGAGAGT AATTAAGTGA 2517

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 784 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met Ala Pro Ser Phe Lys Asn Gly Gly Ser Asn Val Val Ser Phe Asp
1 5 10 15
Gly Leu Asn Asp Met Ser Ser Pro Phe Ala Ile Asp Gly Ser Asp Phe
20 25 30
Thr Val Asn Gly His Ser Phe Leu Ser Asp Val Pro Glu Asn Ile Val
35 40 45
Ala Ser Pro Ser Pro Tyr Thr Ser Ile Asp Lys Ser Pro Val Ser Val
50 55 60
Gly Cys Phe Val Gly Phe Asp Ala Ser Glu Pro Asp Ser Arg His Val
65 70 75 80
Val Ser Ile Gly Lys Leu Lys Asp Ile Arg Phe Met Ser Ile Phe Arg
85 90 95
Phe Lys Val Trp Trp Thr Thr His Trp Val Gly Arg Asn Gly Gly Asp
100 105 110
Leu Glu Ser Glu Thr Gln Ile Val Ile Leu Glu Lys Ser Asp Ser Gly
115 120 125
Arg Pro Tyr Val Phe Leu Leu Pro Ile Val Glu Gly Pro Phe Arg Thr
130 135 140
Ser Ile Gln Pro Gly Asp Asp Phe Val Asp Val Cys Val Glu Ser
145 150 155 160
Gly Ser Ser Lys Val Val Asp Ala Ser Phe Arg Ser Met Leu Tyr Leu
165 170 175
His Ala Gly Asp Asp Pro Phe Ala Leu Val Lys Glu Ala Met Lys Ile
180 185 190
Val Arg Thr His Leu Gly Thr Phe Arg Leu Leu Glu Glu Lys Thr Pro
195 200 205
Pro Gly Ile Val Asp Lys Phe Gly Trp Cys Thr Trp Asp Ala Phe Tyr
210 215 220
Leu Thr Val His Pro Gln Gly Val Ile Glu Gly Val Arg His Leu Val
225 230 235 240
Asp Gly Gly Cys Pro Pro Gly Leu Val Leu Ile Asp Asp Gly Trp Gln
245 250 255
Ser Ile Gly His Asp Ser Asp Pro Ile Thr Lys Glu Gly Met Asn Gln

260	265	270
Thr Val Ala Gly Glu Gln Met Pro Cys Arg Leu Leu Lys Phe Gln Glu		
275	280	285
Asn Tyr Lys Phe Arg Asp Tyr Val Asn Pro Lys Ala Thr Gly Pro Arg		
290	295	300
Ala Gly Gln Lys Gly Met Lys Ala Phe Ile Asp Glu Leu Lys Gly Glu		
305	310	315
Phe Lys Thr Val Glu His Val Tyr Val Trp His Ala Leu Cys Gly Tyr		
325	330	335
Trp Gly Gly Leu Arg Pro Gln Val Pro Gly Leu Pro Glu Ala Arg Val		
340	345	350
Ile Gln Pro Val Leu Ser Pro Gly Leu Gln Met Thr Met Glu Asp Leu		
355	360	365
Ala Val Asp Lys Ile Val Leu His Lys Val Gly Leu Val Pro Pro Glu		
370	375	380
Lys Ala Glu Glu Met Tyr Glu Gly Leu His Ala His Leu Glu Lys Val		
385	390	395
Gly Ile Asp Gly Val Lys Ile Asp Val Ile His Leu Leu Glu Met Leu		
405	410	415
Cys Glu Asp Tyr Gly Gly Arg Val Asp Leu Ala Lys Ala Tyr Tyr Lys		
420	425	430
Ala Met Thr Lys Ser Ile Asn Lys His Phe Lys Gly Asn Gly Val Ile		
435	440	445
Ala Ser Met Glu His Cys Asn Asp Phe Met Phe Leu Gly Thr Glu Ala		
450	455	460
Ile Ser Leu Gly Arg Val Gly Asp Asp Phe Trp Cys Thr Asp Pro Ser		
465	470	475
Gly Asp Pro Asn Gly Thr Phe Trp Leu Gln Gly Cys His Met Val His		
485	490	495
Cys Ala Asn Asp Ser Leu Trp Met Gly Asn Phe Ile His Pro Asp Trp		
500	505	510
Asp Met Phe Gln Ser Thr His Pro Cys Ala Ala Phe His Ala Ala Ser		
515	520	525
Arg Ala Ile Ser Gly Gly Pro Ile Tyr Val Ser Asp Ser Val Gly Lys		
530	535	540
His Asn Phe Asp Leu Leu Lys Lys Leu Val Leu Pro Asp Gly Ser Ile		
545	550	555
Leu Arg Ser Glu Tyr Tyr Ala Leu Pro Thr Arg Asp Cys Leu Phe Glu		
565	570	575
Asp Pro Leu His Asn Gly Glu Thr Met Leu Lys Ile Trp Asn Leu Asn		
580	585	590
Lys Phe Thr Gly Val Ile Gly Ala Phe Asn Cys Gln Gly Gly Gly Trp		
595	600	605
Cys Arg Glu Thr Arg Arg Asn Gln Cys Phe Ser Gln Tyr Ser Lys Arg		
610	615	620

Val Thr Ser Lys Thr Asn Pro Lys Asp Ile Glu Trp His Ser Gly Glu
625 630 635 640
Asn Pro Ile Ser Ile Glu Gly Val Lys Thr Phe Ala Leu Tyr Leu Tyr
645 650 655
Gln Ala Lys Lys Leu Ile Leu Ser Lys Pro Ser Gln Asp Leu Asp Ile
660 665 670
Ala Leu Asp Pro Phe Glu Phe Glu Leu Ile Thr Val Ser Pro Val Thr
675 680 685
Lys Leu Ile Gln Thr Ser Leu His Phe Ala Pro Ile Gly Leu Val Asn
690 695 700
Met Leu Asn Thr Ser Gly Ala Ile Gln Ser Val Asp Tyr Asp Asp Asp
705 710 715 720
Leu Ser Ser Val Glu Ile Gly Val Lys Gly Cys Gly Glu Met Arg Val
725 730 735
Phe Ala Ser Lys Lys Pro Arg Ala Cys Arg Ile Asp Gly Glu Asp Val
740 745 750
Gly Phe Lys Tyr Asp Gln Asp Gln Met Val Val Val Gln Val Pro Trp
755 760 765
Pro Ile Asp Ser Ser Ser Gly Gly Ile Ser Val Ile Glu Tyr Leu Phe
770 775 780

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc= "Synthetic DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TTYTAYCTBA CHGTNCAYCC TCA

23

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc= "Synthetic DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TTYTAYCTBA CHGTNCAYCC CCA

23

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc= "Synthetic DNA"
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TTYTAYCTBA CHGTNCAYCC ACA

23

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc= "Synthetic DNA"
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TTYTAYCTBA CHGTNCAYCC GCA

23

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc= "Synthetic DNA"
- (ix) FEATURE:
 - (A) NAME/KEY:
 - (B) LOCATION:
 - (D) OTHER INFORMATION: N at 6 and 11 = inosine
Other N = A, G, C, or T
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GARGGNGTNM GNCAYCTRGT NGAYGG

26

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc= "Synthetic DNA"
- (ix) FEATURE:

- (A) NAME/KEY:
- (B) LOCATION:
- (D) OTHER INFORMATION: N at 6 and 11 = inosine
Other N = A, G, C, or T

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GARGGNGTNM GNCAYCTYGT NGAYGG

26

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 26 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc= "Synthetic DNA"

(ix) FEATURE:

- (A) NAME/KEY:
- (B) LOCATION:
- (D) OTHER INFORMATION: N at 6 and 11 = inosine
Other N = A, G, C, or T

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GARGGNGTNM GNCAYTTRGT NGAYGG

26

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 26 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc= "Synthetic DNA"
- (ix) FEATURE:
 - (A) NAME/KEY:
 - (B) LOCATION:
 - (D) OTHER INFORMATION: N at 3 = inosine
Other N = A, G, C, or T

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GTNGGNTGYT TYGTNGGYTT YGAYGC

26

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 26 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc= "Synthetic DNA"
- (ix) FEATURE:
 - (A) NAME/KEY:
 - (B) LOCATION:
 - (D) OTHER INFORMATION: N at 3 = inosine
Other N = A, G, C, or T

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GTNGGNTGYT TYGTNGGRTT YGAYGC

26

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 29 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc= "Synthetic DNA"
- (ix) FEATURE:
 - (A) NAME/KEY:
 - (B) LOCATION:
 - (D) OTHER INFORMATION: N at 9 and 11 = inosine
Other N = A, G, C, or T

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

TTYGAYGCNT CNGARCCHGA YTCDCGNCA

29

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc= "Synthetic DNA"
- (ix) FEATURE:
 - (A) NAME/KEY:
 - (B) LOCATION:
 - (D) OTHER INFORMATION: N at 9 and 11 = inosine
Other N = A, G, C, or T

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TTYGAYGCNT CNGARCCHGA YTCDAGYCA

30

(2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs

- (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc= "Synthetic DNA"
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GAYCARGAYC TRATGGTNGT

20

(2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 26 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc= "Synthetic DNA"
- (ix) FEATURE:
 - (A) NAME/KEY:
 - (B) LOCATION:
 - (D) OTHER INFORMATION: N at 6 and 15 = inosine
Other N = A, G, C, or T

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CCRTCNACYA GRTGNCKNAC NCCYTC

26

(2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 26 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc= "Synthetic DNA"
- (ix) FEATURE:
 - (A) NAME/KEY:
 - (B) LOCATION:
 - (D) OTHER INFORMATION: N at 6 and 15 = inosine
Other N = A, G, C, or T

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CCRTCNACRA GRTGNCKNAC NCCYTC

26

(2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 26 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single

- (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc= "Synthetic DNA"
- (ix) FEATURE:
 - (A) NAME/KEY:
 - (B) LOCATION:
 - (D) OTHER INFORMATION: N at 6 and 15 = inosine
Other N = A, G, C, or T
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CCRTCNACYA TRTGNCCKNAC NCCYTC

26

(2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 29 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc= "Synthetic DNA"
- (ix) FEATURE:
 - (A) NAME/KEY:
 - (B) LOCATION:
 - (D) OTHER INFORMATION: N at 3 and 18 = inosine
Other N = A, G, C, or T
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

TGNCGHGART CDGGYTCNGA NGCRTCAA

29

(2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc= "Synthetic DNA"
- (ix) FEATURE:
 - (A) NAME/KEY:
 - (B) LOCATION:
 - (D) OTHER INFORMATION: N at 19 = inosine
Other N = A, G, C, or T
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

RTGRCTHGAR TCDGGYTCNG ANGRTCRAA

30

What is claimed is:

1. A raffinose synthase which has the following properties:

(1) action and substrate specificity: the raffinose synthase produces raffinose from sucrose and galactinol;

(2) optimum pH: the raffinose synthase has an optimum pH of about 6 to 8;

(3) optimum temperature: the raffinose synthase has an optimum temperature of about 35 to 40 °C;

(4) molecular weight: the raffinose synthase has:

(i) a molecular weight of about 75 kDa to 95 kDa estimated by gel filtration chromatography;

(ii) a molecular weight of about 90 kDa to 100 kDa estimated by polyacrylamide gel electrophoresis; and

(iii) a molecular weight of about 90 kDa to 100 kDa estimated by SDS-polyacrylamide gel electrophoresis under a reduced condition;

(5) inhibition: the raffinose synthase is inhibited by iodoacetamide, N-ethylmaleimide, and myo-inositol.

2. The raffinose synthase according to claim 1, which has an amino acid sequence including respective amino acid sequences shown in SEQ ID NOs. 1 to 3 in Sequence Listing.

3. A raffinose synthase which is a protein specified by the following item (A) or (B):

(A) a protein which has an amino acid sequence shown in SEQ ID NO: 5 in Sequence Listing; or

(B) a protein which comprises an amino acid sequence including substitution, deletion, insertion, addition, or inversion of one or several residues of amino acids in the amino acid sequence shown in SEQ ID NO: 5 in Sequence Listing, and which has an activity to produce raffinose from sucrose and galactinol.

4. A method for producing raffinose, comprising the step of allowing the raffinose synthase as defined in any one of claims 1 to 3 to act on sucrose and galactinol to produce raffinose.

5. DNA encoding raffinose synthase as defined in any one of claims 1 to 3.

6. DNA coding for a protein specified by the following item (A) or (B):

(A) a protein which has an amino acid sequence shown in SEQ ID NO: 5 in Sequence Listing; or

(B) a protein which comprises an amino acid sequence including substitution, deletion, insertion, addition, or inversion of one or several residues of amino acids in the amino acid sequence shown in SEQ ID NO: 5 in Sequence Listing, and which has an activity to produce raffinose from sucrose and galactinol.

7. The DNA according to claim 5, which is DNA specified by the following item (a) or (b):

(a) DNA which includes a nucleotide sequence

comprising at least nucleotide residues having nucleotide numbers of 57 to 2408 in a nucleotide sequence shown in SEQ ID NO: 4 in Sequence Listing; or

(b) DNA which is hybridizable under a stringent condition with the nucleotide sequence comprising at least nucleotide residues having nucleotide numbers of 57 to 2408 in the nucleotide sequence shown in SEQ ID NO: 4 in Sequence Listing, and which codes for a protein having an activity to produce raffinose from sucrose and galactinol.

8. A chimeric gene comprising a raffinose synthase gene or a part thereof, and a transcription regulatory region expressible in plant cells.

9. The chimeric gene according to claim 8, wherein the raffinose synthase gene is DNA as defined in any one of claims 5 to 7.

10. The chimeric gene according to claim 8 or 9, wherein the transcription regulatory region is ligated with the DNA so that antisense RNA having a sequence complementary to a coding strand of the DNA is expressed.

11. A plant which is transformed with the chimeric gene as defined in any one of claims 8 to 10.

12. A method for changing a content of raffinose family oligosaccharides in a plant, comprising the steps of transforming the plant with the chimeric gene as defined in any one of claims 8 to 10, and expressing the

gene in cells of the plant.

Abstract

Raffinose synthase purified from cucumber is allowed to act on sucrose and galactinol. Thus raffinose is efficiently produced. The function of endogenous raffinose synthase is regulated by transforming a plant with a chimeric gene comprising a raffinose synthase gene and a regulatory region expressible in the plant. Thus a plant, in which raffinose family oligosaccharides are decreased, is created.

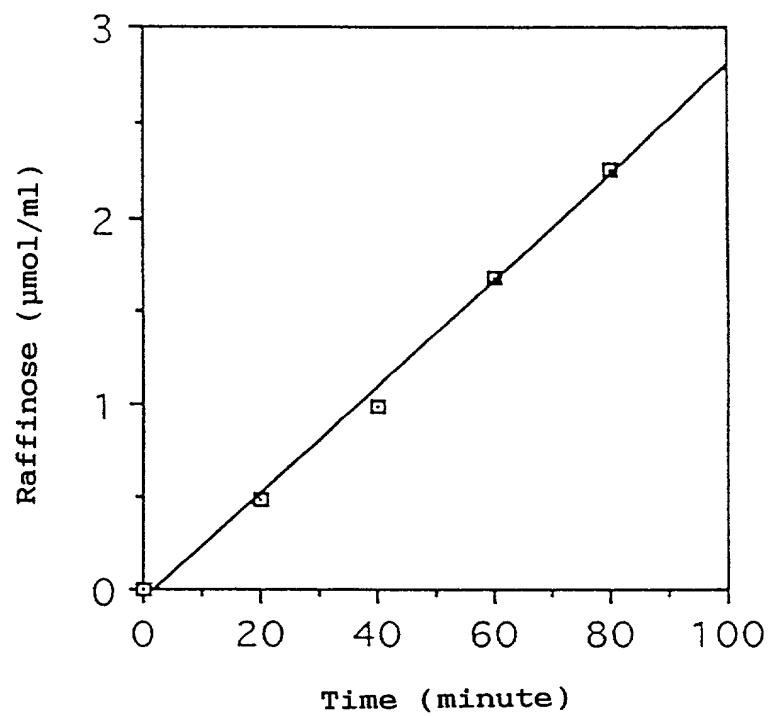
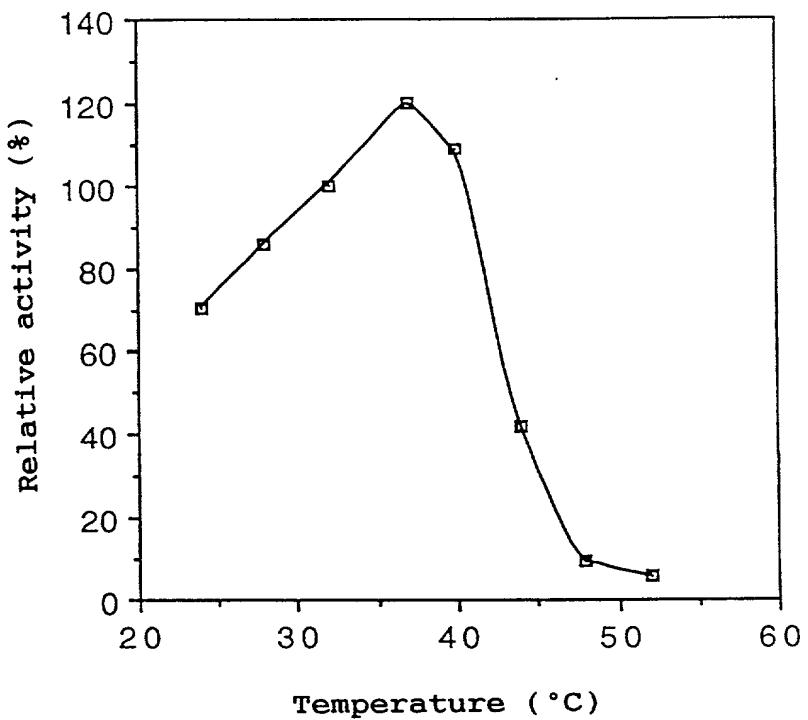


FIG. 1

M S

200	—
116	■
97.4	■
66	■
45	■

F I G. 2



F / G. 3

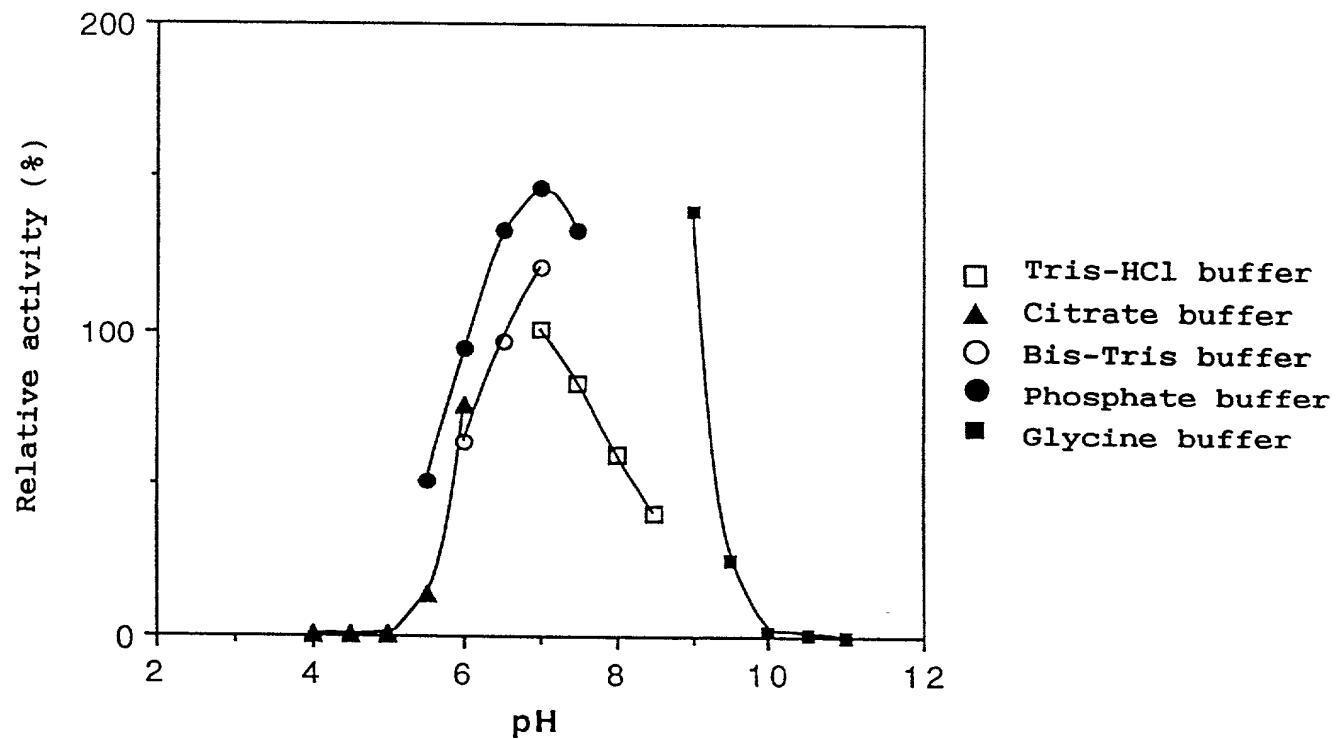
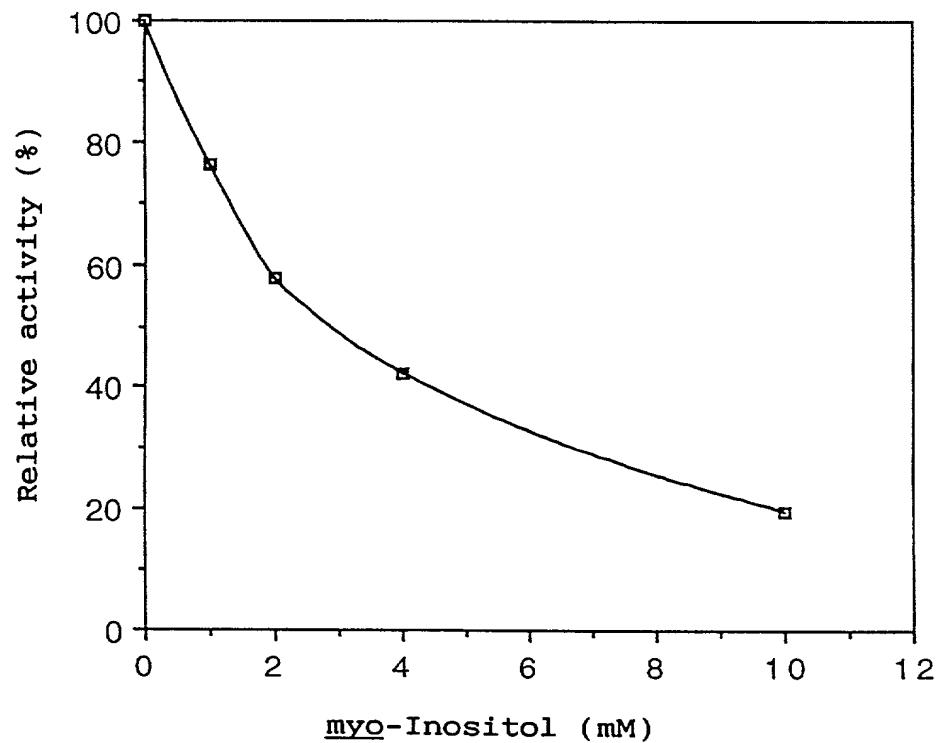


FIG. 4



F / G. 5

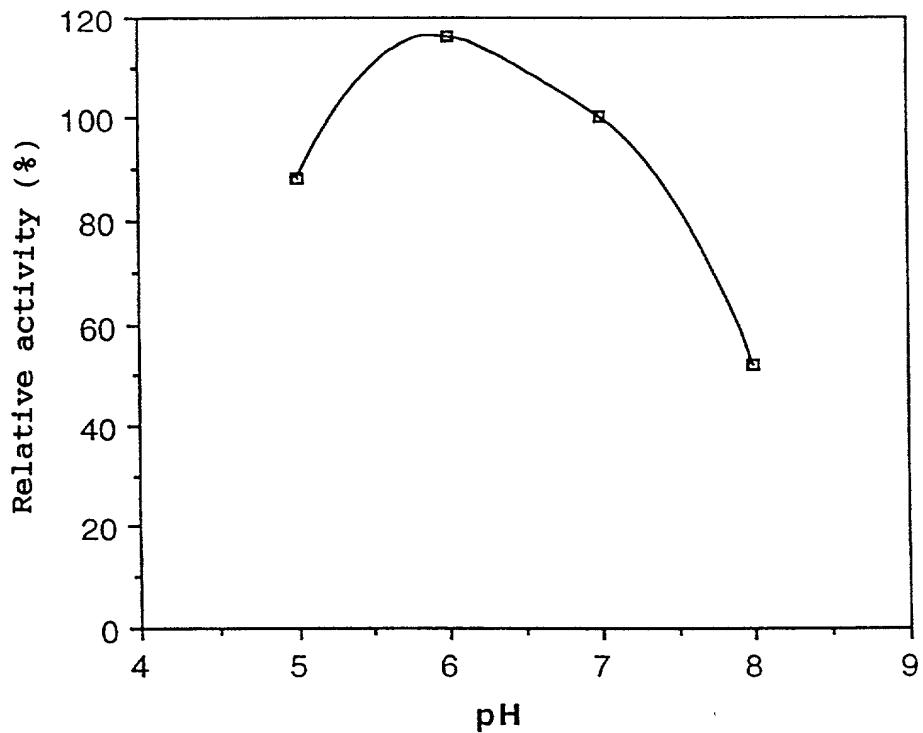


FIG. 6

(SEQ ID NO:6) A1 5' - TTY TAY CTB ACH GTN CAY CCT CA -3'
(SEQ ID NO:7) A2 5' - TTY TAY CTB ACH GTN CAY CCC CA -3' B1 5' - GAR GGN GTN MGN CAY CTR GTN GAY GG -3' (SEQ ID NO:10)
(SEQ ID NO:8) A3 5' - TTY TAY CTB ACH GTN CAY CCA CA -3' B2 5' - GAR GGN GTN MGN CAY CTR GTN GAY GG -3' (SEQ ID NO:11)
(SEQ ID NO:9) A4 5' - TTY TAY CTB ACH GTN CAY CCG CA -3' B3 5' - GAR GGN GTN MGN CAY TTR GTN GAY GG -3' (SEQ ID NO:12)
Phe Gly Trp Cys Thr Trp Asp Ala Phe Tyr Leu Thr Val His Pro Gln Gly Val Ile Glu Val Arg His Leu Val Asp Gly Gly Cys
(SEQ ID NO:1)

(SEQ ID NO:18) 3' - CTY CCN CAN KCI GTR GAY CAI CTR CC -5' B' 1
(SEQ ID NO:19) 3' - CTY CCN CAN KCI GTR GAR CAI CTR CC -5' B' 2
(SEQ ID NO:20) 3' - CTY CCN CAN KCI GTR TAY CAI CTR CC -5' B' 3

D1 5' - TTY GAY GCN TCN GAR CCH GAY TCD CGN CA -3' (SEQ ID NO:15)
D2 5' - TTY GAY GCN TCN GAR CCH GAY TCD AGY CAY -3' (SEQ ID NO:16)

C1 5' - GTN GGN TGY TTY GTN GGY TTY GAY GC -3' (SEQ ID NO:13)

C2 5' - GTN GGN TGY TTY GTN GGR TTY GAY GC -3' (SEQ ID NO:14)

Pro Val Ser Val Gly Cys Phe Val Gly Phe Asp Ala Ser Glu Pro Asp Ser Arg His
(SEQ ID NO:2) 3' - AAR CTR CGN AGI CTR GGD CTR AGH GCI GT -5' D' 1 (SEQ ID NO:21)
3' - AAR CTR CGN AGI CTR GGD CTR AGH TCR GTR -5' D' 2 (SEQ ID NO:22)

E 5' - GAY CAR GAY CTR ATG GTN GT -3' (SEQ ID NO:17)
Tyr Asp Gln Asp Gln Met Val Val Gln Val Pro Trp Pro
(SEQ ID NO:3)

Declaration, Power Of Attorney and Petition

Page 1 of 3

WE (I) the undersigned inventor(s), hereby declare(s) that:

My residence, post office address and citizenship are as stated below next to my name,

We (I) believe that we are (I am) the original, first, and joint (sole) inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention entitled

RAFFINOSE SYNTHASE GENE, METHOD FOR PRODUCING

RAFFINOSE, AND TRANSGENIC PLANT

the specification of which

is attached hereto.

was filed on April 28, 1997 as

Application Serial No. 08/846,234

and amended on _____.

was filed as PCT international application

Number _____

on _____,

and was amended under PCT Article 19

on _____ (if applicable).

We (I) hereby state that we (I) have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

We (I) acknowledge the duty to disclose information known to be material to the patentability of this application as defined in Section 1.56 of Title 37 Code of Federal Regulations.

We (I) hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed. Prior Foreign Application(s)

Application No.	Country	Day/Month/Year	Priority Claimed
8-107682	Japan	26/04/1996	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No
8-198079	Japan	26/07/1996	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No
_____	_____	_____	<input type="checkbox"/> Yes <input type="checkbox"/> No
_____	_____	_____	<input type="checkbox"/> Yes <input type="checkbox"/> No

We (I) hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below.

(Application Number)	(Filing Date)
(Application Number)	(Filing Date)

We (I) hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or § 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

Application Serial No.	Filing Date	Status (pending, patented, abandoned)

And we (I) hereby appoint Norman F. Oblon, Registration Number 24,618; Marvin J. Spivak, Registration Number 24,913; C. Irvin McClelland, Registration Number 21,124; Gregory J. Maier, Registration Number 25,599; Arthur I. Neustadt, Registration Number 24,854; Richard D. Kelly, Registration Number 27,757; James D. Hamilton, Registration Number 28,421; Eckhard H. Kuesters, Registration Number 28,870; Robert T. Pous, Registration Number 29,099; Charles L. Gholz, Registration Number 26,395; Vincent J. Sunderdick, Registration Number 29,004; William E. Beaumont, Registration Number 30,996; Steven B. Kelber, Registration Number 30,073; Robert F. Gnuse, Registration Number 27,295; Jean-Paul Lavallee, Registration Number 31,451; Timothy R. Schwartz, Registration Number 32,171; Stephen G. Baxter, Registration Number 32,884; Martin M. Zoltick, Registration Number 35,745; Robert W. Hahl, Registration Number 33,893; Richard L. Treanor, Registration Number 36,379; Steven P. Weihrouch, Registration Number 32,829; John T. Gookasian, Registration Number 26,142; Marc R. Labgold, Registration Number 34,651; William J. Healey, Registration Number 36,160; Richard L. Chinn, Registration Number 34,305; Steven E. Lipman, Registration Number 30,011; Carl E. Schlier, Registration Number 34,426; James J. Kulbaski, Registration Number 34,648; Catherine B. Richardson, Registration Number 39,007; Richard A. Neifeld, Registration Number 35,299; J. Derek Mason, Registration Number 35,270; and Jacques M. Dulin, Registration Number 24,067; our (my) attorneys, with full powers of substitution and revocation, to prosecute this application and to transact all business in the Patent Office connected therewith; and we (I) hereby request that all correspondence regarding this application be sent to the firm of OBLON, SPIVAK, McCLELLAND, MAIER & NEUSTADT, P.C., whose Post Office Address is: Fourth Floor, 1755 Jefferson Davis Highway, Arlington, Virginia 22202.

We (I) declare that all statements made herein of our (my) own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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Signature of Inventor

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Date

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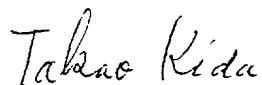
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Signature of Inventor

September 3, 1997
Date

Takao Kida
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Signature of Inventor

September 3, 1997
Date

NAME OF FOURTH JOINT INVENTOR

Signature of Inventor

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NAME OF FIFTH JOINT INVENTOR

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Citizen of: Japan

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Residence:

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Post Office Address:

Residence:

Citizen of:

Post Office Address:

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: OSUMI Chieko
NOZAKI Jinshi
KIDA Takao
- (ii) TITLE OF INVENTION: RAFFINOSE SYNTHASE GENE, METHOD FOR PRODUCING RAFFINOSE, AND TRANSGENIC PLANT
- (iii) NUMBER OF SEQUENCES: 22
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: OBLON, SPIVAK, MCCLELLAND, MAIER & NEUSTADT, P.C.
 - (B) STREET: 1755 S. JEFFERSON DAVIS HIGHWAY, FOURTH FLOOR
 - (C) CITY: ARLINGTON
 - (D) STATE: VIRGINIA
 - (E) COUNTRY: USA
 - (F) ZIP: 22202
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)
- (vi) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: NORMAN F. OBLON
 - (B) REGISTRATION NUMBER: 24,618
- (vii) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (703)-413-3000
 - (B) TELEFAX: (703)-413-2220

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (v) FRAGMENT TYPE: internal
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Phe Gly Trp Cys Thr Trp Asp Ala Phe Tyr Leu Thr Val His Pro Gln
1 5 10 15
Gly Val Ile Glu Gly Val Arg His Leu Val Asp Gly Gly Cys
20 25 30

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 19 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Pro Val Ser Val Gly Cys Phe Val Gly Phe Asp Ala Ser Glu Pro Asp
1 5 10 15
Ser Arg His

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Tyr Asp Gln Asp Gln Met Val Val Val Gln Val Pro Trp Pro
1 5 10

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2517 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: cucumber (*Cucumis sativas*)

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 56..2407

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AAAAAAACAAC CCTTCTTTA GTTTTTGGG TTTGTTCTT CTTTCTTCT CACAA ATG 58
Met
1
GCT CCT AGT TTT AAA AAT GGT GGC TCC AAC GTA GTT TCA TTT GAT GGC 106
Ala Pro Ser Phe Lys Asn Gly Gly Ser Asn Val Val Ser Phe Asp Gly
5 10 15
TTA AAT GAC ATG TCG TCA CCG TTT GCA ATC GAC GGA TCG GAT TTC ACT 154
Leu Asn Asp Met Ser Ser Pro Phe Ala Ile Asp Gly Ser Asp Phe Thr
20 25 30
GTG AAC GGT CAT TCG TTT CTG TCC GAT GTT CCT GAG AAC ATT GTT GCT 202
Val Asn Gly His Ser Phe Leu Ser Asp Val Pro Glu Asn Ile Val Ala
35 40 45
TCT CCT TCT CCG TAC ACT TCG ATA GAC AAG TCC CCG GTT TCG GTT GGT 250
Ser Pro Ser Pro Tyr Thr Ser Ile Asp Lys Ser Pro Val Ser Val Gly
50 55 60 65
TGC TTT GTT GGA TTC GAC GCG TCG GAA CCT GAT AGC CGA CAT GTT GTT 298
Cys Phe Val Gly Phe Asp Ala Ser Glu Pro Asp Ser Arg His Val Val
70 75 80
TCG ATT GGG AAG CTG AAG GAT ATT CGG TTT ATG AGT ATT TTC AGG TTT 346
Ser Ile Gly Lys Leu Lys Asp Ile Arg Phe Met Ser Ile Phe Arg Phe

85	90	95	
AAG GTT TGG TGG ACT ACA CAC TGG GTT GGT CGA AAT GGT GGG GAT CTT			394
Lys Val Trp Trp Thr Thr His Trp Val Gly Arg Asn Gly Gly Asp Leu			
100 105 110			
GAA TCG GAG ACT CAG ATT GTG ATC CTT GAG AAG TCA GAT TCT GGT CGA			442
Glu Ser Glu Thr Gln Ile Val Ile Leu Glu Lys Ser Asp Ser Gly Arg			
115 120 125			
CCG TAT GTT TTC CTT CTT CCG ATC GTT GAG GGA CCG TTC CGA ACC TCG			490
Pro Tyr Val Phe Leu Leu Pro Ile Val Glu Gly Pro Phe Arg Thr Ser			
130 135 140 145			
ATT CAG CCT GGG GAT GAT GAC TTT GTC GAT GTT TGT GTC GAG AGT GGT			538
Ile Gln Pro Gly Asp Asp Asp Phe Val Asp Val Cys Val Glu Ser Gly			
150 155 160			
TCG TCG AAA GTT GTT GAT GCA TCG TTC CGA AGT ATG TTG TAT CTT CAT			586
Ser Ser Lys Val Val Asp Ala Ser Phe Arg Ser Met Leu Tyr Leu His			
165 170 175			
GCT GGT GAT GAT CCG TTT GCA CTT GTT AAA GAG GCG ATG AAG ATC GTG			634
Ala Gly Asp Asp Pro Phe Ala Leu Val Lys Glu Ala Met Lys Ile Val			
180 185 190			
AGG ACC CAT CTT GGA ACT TTT CGC TTG TTG GAG GAG AAG ACT CCA CCA			682
Arg Thr His Leu Gly Thr Phe Arg Leu Leu Glu Glu Lys Thr Pro Pro			
195 200 205			
GGT ATC GTG GAC AAA TTC GGT TGG TGC ACG TGG GAC GCG TTT TAC CTA			730
Gly Ile Val Asp Lys Phe Gly Trp Cys Thr Trp Asp Ala Phe Tyr Leu			
210 215 220 225			
ACG GTT CAT CCA CAG GGC GTA ATA GAA GGC GTG AGG CAT CTC GTC GAC			778
Thr Val His Pro Gln Gly Val Ile Glu Gly Val Arg His Leu Val Asp			
230 235 240			
GGC GGT TGT CCT CCC GGT TTA GTC CTA ATC GAC GAT GGT TGG CAA TCC			826
Gly Gly Cys Pro Pro Gly Leu Val Leu Ile Asp Asp Gly Trp Gln Ser			
245 250 255			
ATC GGA CAC GAT TCG GAT CCC ATC ACC AAA GAA GGA ATG AAC CAA ACC			874
Ile Gly His Asp Ser Asp Pro Ile Thr Lys Glu Gly Met Asn Gln Thr			
260 265 270			
GTC GCC GGC GAG CAA ATG CCC TGC CGT CTT TTG AAA TTC CAA GAG AAT			922
Val Ala Gly Glu Gln Met Pro Cys Arg Leu Leu Lys Phe Gln Glu Asn			
275 280 285			
TAC AAA TTC CGT GAC TAC GTC AAT CCC AAG GCC ACC GGC CCC CGA GCC			970
Tyr Lys Phe Arg Asp Tyr Val Asn Pro Lys Ala Thr Gly Pro Arg Ala			
290 295 300 305			
GGC CAG AAG GGG ATG AAG GCG TTT ATA GAT GAA CTC AAA GGA GAG TTT			1018
Gly Gln Lys Gly Met Lys Ala Phe Ile Asp Glu Leu Lys Gly Glu Phe			
310 315 320			
AAG ACT GTG GAG CAT GTT TAT GTT TGG CAT GCT TTG TGT GGA TAT TGG			1066
Lys Thr Val Glu His Val Tyr Val Trp His Ala Leu Cys Gly Tyr Trp			
325 330 335			
GGT GGC CTT CGC CCG CAG GTG CCT GGC TTG CCT GAG GCA CGT GTG ATT			1114
Gly Gly Leu Arg Pro Gln Val Pro Gly Leu Pro Glu Ala Arg Val Ile			
340 345 350			

CAG CCA GTG CTT TCA CCA GGG CTG CAG ATG ACG ATG GAG GAT TTG GCG	1162
Gln Pro Val Leu Ser Pro Gly Leu Gln Met Thr Met Glu Asp Leu Ala	
355 360 365	
GTG GAT ATT GTT CTT CAT AAG GTC GGG CTG GTC CCG CCG GAG AAG	1210
Val Asp Lys Ile Val Leu His Lys Val Gly Leu Val Pro Pro Glu Lys	
370 375 380 385	
GCT GAG GAG ATG TAC GAA GGA CTT CAT GCT CAT TTG GAA AAA GTT GGG	1258
Ala Glu Glu Met Tyr Glu Gly Leu His Ala His Leu Glu Lys Val Gly	
390 395 400	
ATC GAC GGT GTT AAG ATT GAC GTT ATC CAC CTA TTG GAG ATG TTG TGT	1306
Ile Asp Gly Val Lys Ile Asp Val Ile His Leu Leu Glu Met Leu Cys	
405 410 415	
GAA GAC TAT GGA GGG AGA GTG GAT TTG GCA AAG GCA TAT TAC AAA GCA	1354
Glu Asp Tyr Gly Gly Arg Val Asp Leu Ala Lys Ala Tyr Tyr Lys Ala	
420 425 430	
ATG ACC AAA TCA ATA AAT AAA CAT TTT AAA GGA AAT GGA GTC ATT GCA	1402
Met Thr Lys Ser Ile Asn Lys His Phe Lys Gly Asn Gly Val Ile Ala	
435 440 445	
AGT ATG GAA CAT TGT AAC GAC TTC ATG TTC CTT GGC ACG GAA GCT ATC	1450
Ser Met Glu His Cys Asn Asp Phe Met Phe Leu Gly Thr Glu Ala Ile	
450 455 460 465	
TCT CTT GGT CGT GTT GGT GAT GAC TTT TGG TGC ACG GAC CCC TCT GGT	1498
Ser Leu Gly Arg Val Gly Asp Asp Phe Trp Cys Thr Asp Pro Ser Gly	
470 475 480	
GAT CCA AAC GGT ACG TTT TGG CTC CAA GGA TGT CAC ATG GTT CAT TGT	1546
Asp Pro Asn Gly Thr Phe Trp Leu Gln Gly Cys His Met Val His Cys	
485 490 495	
GCC AAC GAC AGC TTG TGG ATG GGG AAC TTC ATC CAC CCT GAC TGG GAT	1594
Ala Asn Asp Ser Leu Trp Met Gly Asn Phe Ile His Pro Asp Trp Asp	
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ATG TTC CAA TCC ACC CAC CCT TGT GCC GCC TTC CAT GCT GCC TCT CGA	1642
Met Phe Gln Ser Thr His Pro Cys Ala Ala Phe His Ala Ala Ser Arg	
515 520 525	
GCC ATC TCT GGT GGC CCG ATC TAT GTT AGT GAT TCT GTG GGA AAG CAT	1690
Ala Ile Ser Gly Gly Pro Ile Tyr Val Ser Asp Ser Val Gly Lys His	
530 535 540 545	
AAC TTT GAT CTT CTG AAA AAA CTA GTG CTT CCT GAT GGA TCG ATC CTT	1738
Asn Phe Asp Leu Leu Lys Lys Leu Val Leu Pro Asp Gly Ser Ile Leu	
550 555 560	
CGA AGT GAG TAC TAT GCA CTC CCG ACT CGC GAT TGT TTG TTT GAA GAC	1786
Arg Ser Glu Tyr Tyr Ala Leu Pro Thr Arg Asp Cys Leu Phe Glu Asp	
565 570 575	
CCT TTG CAT AAT GGA GAA ACT ATG CTT AAG ATT TGG AAT CTC AAC AAG	1834
Pro Leu His Asn Gly Glu Thr Met Leu Lys Ile Trp Asn Leu Asn Lys	
580 585 590	
TTC ACT GGA GTG ATT GGT GCA TTC AAC TGC CAA GGA GGA GGA TGG TGT	1882
Phe Thr Gly Val Ile Gly Ala Phe Asn Cys Gln Gly Gly Gly Trp Cys	
595 600 605	
CGT GAG ACA CGA CGC AAC CAA TGC TTT TCA CAA TAC TCA AAA CGA GTG	1930

Arg	Glu	Thr	Arg	Arg	Asn	Gln	Cys	Phe	Ser	Gln	Tyr	Ser	Lys	Arg	Val	
610				615						620					625	
ACA	TCC	AAA	ACT	AAC	CCA	AAA	GAC	ATA	GAA	TGG	CAC	AGT	GGA	GAA	AAC	1978
Thr	Ser	Lys	Thr	Asn	Pro	Lys	Asp	Ile	Glu	Trp	His	Ser	Gly	Glu	Asn	
					630				635					640		
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Pro	Ile	Ser	Ile	Glu	Gly	Val	Lys	Thr	Phe	Ala	Leu	Tyr	Leu	Tyr	Gln	
					645				650					655		
GCC	AAA	AAA	CTT	ATC	CTC	TCC	AAG	CCC	TCT	CAA	GAT	CTT	GAC	ATA	GCT	2074
Ala	Lys	Lys	Leu	Ile	Leu	Ser	Lys	Pro	Ser	Gln	Asp	Leu	Asp	Ile	Ala	
					660				665					670		
CTT	GAC	CCA	TTC	GAA	TTC	GAG	CTC	ATC	ACT	GTT	TCA	CCA	GTG	ACC	AAA	2122
Leu	Asp	Pro	Phe	Glu	Phe	Glu	Leu	Ile	Thr	Val	Ser	Pro	Val	Thr	Lys	
					675				680					685		
CTC	ATC	CAA	ACT	TCT	CTA	CAC	TTT	GCC	CCA	ATT	GGG	CTG	GTG	AAC	ATG	2170
Leu	Ile	Gln	Thr	Ser	Leu	His	Phe	Ala	Pro	Ile	Gly	Leu	Val	Asn	Met	
					690				695					700		705
CTT	AAC	ACT	AGT	GGA	GCC	ATC	CAA	TCT	GTG	GAC	TAT	GAC	GAT	GAC	CTA	2218
Leu	Asn	Thr	Ser	Gly	Ala	Ile	Gln	Ser	Val	Asp	Tyr	Asp	Asp	Asp	Leu	
					710				715					720		
AGC	TCA	GTC	GAG	ATT	GGT	GTC	AAA	GGG	TGT	GGT	GAG	ATG	CGA	GTA	TTT	2266
Ser	Ser	Val	Glu	Ile	Gly	Val	Lys	Gly	Cys	Gly	Glu	Met	Arg	Val	Phe	
					725				730					735		
GCA	TCG	AAA	AAA	CCA	AGG	GCT	TGT	CGT	ATT	GAT	GGG	GAG	GAT	GTT	GGG	2314
Ala	Ser	Lys	Lys	Pro	Arg	Ala	Cys	Arg	Ile	Asp	Gly	Glu	Asp	Val	Gly	
					740				745					750		
TTC	AAG	TAT	GAT	CAG	GAC	CAA	ATG	GTG	GTG	GTT	CAA	GTG	CCA	TGG	CCA	2362
Phe	Lys	Tyr	Asp	Gln	Asp	Gln	Met	Val	Val	Val	Gln	Val	Pro	Trp	Pro	
					755				760					765		
ATT	GAT	TCT	TCA	TCG	GGT	GGC	ATT	TCG	GTT	ATC	GAG	TAC	TTG	TTT		2407
Ile	Asp	Ser	Ser	Ser	Gly	Gly	Ile	Ser	Val	Ile	Glu	Tyr	Leu	Phe		
					770				775					780		
TAATTTTAT	TTATGTAAGC	TCAATGATTG	TTGTTGTTGT	CGCTGTTGTT	GCTATCAATG											2467
TATTTCTCTC	CAAAAGAAAA	TTATGTGTA	TTGGAGAGT	AATTAAGTGA												2517

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 784 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met	Ala	Pro	Ser	Phe	Lys	Asn	Gly	Gly	Ser	Asn	Val	Val	Ser	Phe	Asp	
1				5					10					15		
Gly	Leu	Asn	Asp	Met	Ser	Ser	Pro	Phe	Ala	Ile	Asp	Gly	Ser	Asp	Phe	
				20					25					30		
Thr	Val	Asn	Gly	His	Ser	Phe	Leu	Ser	Asp	Val	Pro	Glu	Asn	Ile	Val	
				35					40					45		
Ala	Ser	Pro	Ser	Pro	Tyr	Thr	Ser	Ile	Asp	Lys	Ser	Pro	Val	Ser	Val	

50	55	60														
Gly	Cys	Phe	Val	Gly	Phe	Asp	Ala	Ser	Glu	Pro	Asp	Ser	Arg	His	Val	
65				70			75								80	
Val	Ser	Ile	Gly	Lys	Leu	Lys	Asp	Ile	Arg	Phe	Met	Ser	Ile	Phe	Arg	
							85		90						95	
Phe	Lys	Val	Trp	Trp	Thr	Thr	His	Trp	Val	Gly	Arg	Asn	Gly	Gly	Asp	
							100		105						110	
Leu	Glu	Ser	Glu	Thr	Gln	Ile	Val	Ile	Leu	Glu	Lys	Ser	Asp	Ser	Gly	
							115		120						125	
Arg	Pro	Tyr	Val	Phe	Leu	Leu	Pro	Ile	Val	Glu	Gly	Pro	Phe	Arg	Thr	
							130		135						140	
Ser	Ile	Gln	Pro	Gly	Asp	Asp	Asp	Phe	Val	Asp	Val	Cys	Val	Glu	Ser	
							145		150						160	
Gly	Ser	Ser	Lys	Val	Val	Asp	Ala	Ser	Phe	Arg	Ser	Met	Leu	Tyr	Leu	
							165		170						175	
His	Ala	Gly	Asp	Asp	Pro	Phe	Ala	Leu	Val	Lys	Glu	Ala	Met	Lys	Ile	
							180		185						190	
Val	Arg	Arg	Thr	His	Leu	Gly	Thr	Phe	Arg	Leu	Leu	Glu	Glu	Lys	Thr	Pro
							195		200						205	
Pro	Gly	Ile	Val	Asp	Lys	Phe	Gly	Trp	Cys	Thr	Trp	Asp	Ala	Phe	Tyr	
							210		215						220	
Leu	Thr	Val	His	Pro	Gln	Gly	Val	Ile	Glu	Gly	Val	Arg	His	Leu	Val	
							225		230						240	
Asp	Gly	Gly	Cys	Pro	Pro	Gly	Leu	Val	Leu	Ile	Asp	Asp	Gly	Trp	Gln	
							245		250						255	
Ser	Ile	Gly	His	Asp	Ser	Asp	Pro	Ile	Thr	Lys	Glu	Gly	Met	Asn	Gln	
							260		265						270	
Thr	Val	Ala	Gly	Glu	Gln	Met	Pro	Cys	Arg	Leu	Leu	Lys	Phe	Gln	Glu	
							275		280						285	
Asn	Tyr	Lys	Phe	Arg	Asp	Tyr	Val	Asn	Pro	Lys	Ala	Thr	Gly	Pro	Arg	
							290		295						300	
Ala	Gly	Gln	Lys	Gly	Met	Lys	Ala	Phe	Ile	Asp	Glu	Leu	Lys	Gly	Glu	
							305		310						320	
Phe	Lys	Thr	Val	Glu	His	Val	Tyr	Val	Trp	His	Ala	Leu	Cys	Gly	Tyr	
							325		330						335	
Trp	Gly	Gly	Leu	Arg	Pro	Gln	Val	Pro	Gly	Leu	Pro	Glu	Ala	Arg	Val	
							340		345						350	
Ile	Gln	Pro	Val	Leu	Ser	Pro	Gly	Leu	Gln	Met	Thr	Met	Glu	Asp	Leu	
							355		360						365	
Ala	Val	Asp	Lys	Ile	Val	Leu	His	Lys	Val	Gly	Leu	Val	Pro	Pro	Glu	
							370		375						380	
Lys	Ala	Glu	Glu	Met	Tyr	Glu	Gly	Leu	His	Ala	His	Leu	Glu	Lys	Val	
							385		390						400	
Gly	Ile	Asp	Gly	Val	Lys	Ile	Asp	Val	Ile	His	Leu	Leu	Glu	Met	Leu	
							405		410						415	
Cys	Glu	Asp	Tyr	Gly	Gly	Arg	Val	Asp	Leu	Ala	Lys	Ala	Tyr	Tyr	Lys	
							420		425						430	
Ala	Met	Thr	Lys	Ser	Ile	Asn	Lys	His	Phe	Lys	Gly	Asn	Gly	Val	Ile	
							435		440						445	

Ala Ser Met Glu His Cys Asn Asp Phe Met Phe Leu Gly Thr Glu Ala
 450 455 460
 Ile Ser Leu Gly Arg Val Gly Asp Asp Phe Trp Cys Thr Asp Pro Ser
 465 470 475 480
 Gly Asp Pro Asn Gly Thr Phe Trp Leu Gln Gly Cys His Met Val His
 485 490 495
 Cys Ala Asn Asp Ser Leu Trp Met Gly Asn Phe Ile His Pro Asp Trp
 500 505 510
 Asp Met Phe Gln Ser Thr His Pro Cys Ala Ala Phe His Ala Ala Ser
 515 520 525
 Arg Ala Ile Ser Gly Gly Pro Ile Tyr Val Ser Asp Ser Val Gly Lys
 530 535 540
 His Asn Phe Asp Leu Leu Lys Leu Val Leu Pro Asp Gly Ser Ile
 545 550 555 560
 Leu Arg Ser Glu Tyr Tyr Ala Leu Pro Thr Arg Asp Cys Leu Phe Glu
 565 570 575
 Asp Pro Leu His Asn Gly Glu Thr Met Leu Lys Ile Trp Asn Leu Asn
 580 585 590
 Lys Phe Thr Gly Val Ile Gly Ala Phe Asn Cys Gln Gly Gly Trp
 595 600 605
 Cys Arg Glu Thr Arg Arg Asn Gln Cys Phe Ser Gln Tyr Ser Lys Arg
 610 615 620
 Val Thr Ser Lys Thr Asn Pro Lys Asp Ile Glu Trp His Ser Gly Glu
 625 630 635 640
 Asn Pro Ile Ser Ile Glu Gly Val Lys Thr Phe Ala Leu Tyr Leu Tyr
 645 650 655
 Gln Ala Lys Lys Leu Ile Leu Ser Lys Pro Ser Gln Asp Leu Asp Ile
 660 665 670
 Ala Leu Asp Pro Phe Glu Phe Glu Leu Ile Thr Val Ser Pro Val Thr
 675 680 685
 Lys Leu Ile Gln Thr Ser Leu His Phe Ala Pro Ile Gly Leu Val Asn
 690 695 700
 Met Leu Asn Thr Ser Gly Ala Ile Gln Ser Val Asp Tyr Asp Asp Asp
 705 710 715 720
 Leu Ser Ser Val Glu Ile Gly Val Lys Gly Cys Gly Glu Met Arg Val
 725 730 735
 Phe Ala Ser Lys Lys Pro Arg Ala Cys Arg Ile Asp Gly Glu Asp Val
 740 745 750
 Gly Phe Lys Tyr Asp Gln Asp Gln Met Val Val Val Gln Val Pro Trp
 755 760 765
 Pro Ile Asp Ser Ser Ser Gly Gly Ile Ser Val Ile Glu Tyr Leu Phe
 770 775 780

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 23 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc= "Synthetic DNA"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:
TTYTAYCTBA CHGTNCAYCC TCA

23

(2) INFORMATION FOR SEQ ID NO:7:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc= "Synthetic DNA"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

23

TTYTAYCTBA CHGTNCAYCC CCA

(2) INFORMATION FOR SEQ ID NO:8:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc= "Synthetic DNA"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

23

TTYTAYCTBA CHGTNCAYCC ACA

(2) INFORMATION FOR SEQ ID NO:9:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc= "Synthetic DNA"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

23

TTYTAYCTBA CHGTNCAYCC GCA

(2) INFORMATION FOR SEQ ID NO:10:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc= "Synthetic DNA"
(ix) FEATURE:
(A) NAME/KEY:
(B) LOCATION:

(D) OTHER INFORMATION: N at 6 and 11 = inosine
Other N = A, G, C, or T

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GARGGNGTNM GNCAYCTRGT NGAYGG

26

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc= "Synthetic DNA"

(ix) FEATURE:

(A) NAME/KEY:

(B) LOCATION:

(D) OTHER INFORMATION: N at 6 and 11 = inosine

Other N = A, G, C, or T

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

26

GARGGNGTNM GNCAYCTYGT NGAYGG

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc= "Synthetic DNA"

(ix) FEATURE:

(A) NAME/KEY:

(B) LOCATION:

(D) OTHER INFORMATION: N at 6 and 11 = inosine

Other N = A, G, C, or T

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

26

GARGGNGTNM GNCAYTTRGT NGAYGG

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc= "Synthetic DNA"

(ix) FEATURE:

(A) NAME/KEY:

(B) LOCATION:

(D) OTHER INFORMATION: N at 3 = inosine

Other N = A, G, C, or T

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

26

GTNGGNTGYT TYGTNGGYTT YGAYGC

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc= "Synthetic DNA"

(ix) FEATURE:

- (A) NAME/KEY:
- (B) LOCATION:
- (D) OTHER INFORMATION: N at 3 = inosine
Other N = A, G, C, or T

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

26

GTNGGNTGYT TYGTNGGRTT YGAYGC

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc= "Synthetic DNA"

(ix) FEATURE:

- (A) NAME/KEY:
- (B) LOCATION:
- (D) OTHER INFORMATION: N at 9 and 11 = inosine
Other N = A, G, C, or T

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

29

TTYGAYGCNT CNGARCCCHGA YTCDCGNCA

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc= "Synthetic DNA"

(ix) FEATURE:

- (A) NAME/KEY:
- (B) LOCATION:
- (D) OTHER INFORMATION: N at 9 and 11 = inosine
Other N = A, G, C, or T

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

30

TTYGAYGCNT CNGARCCCHGA YTCDAGYCYAY

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc= "Synthetic DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

20

GAYCARGAYC TRATGGTNGT

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc= "Synthetic DNA"

(ix) FEATURE:

- (A) NAME/KEY:
- (B) LOCATION:
- (D) OTHER INFORMATION: N at 6 and 15 = inosine
Other N = A, G, C, or T

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

26

CCRTCNACYA GRTGNCKNAC NCCYTC

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc= "Synthetic DNA"

(ix) FEATURE:

- (A) NAME/KEY:
- (B) LOCATION:
- (D) OTHER INFORMATION: N at 6 and 15 = inosine
Other N = A, G, C, or T

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

26

CCRTCNACRA GRTGNCKNAC NCCYTC

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc= "Synthetic DNA"
(ix) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(D) OTHER INFORMATION: N at 6 and 15 = inosine
Other N = A, G, C, or T
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CCRTCNACYA TRTGNCKNAC NCCYTC

26

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc= "Synthetic DNA"
(ix) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(D) OTHER INFORMATION: N at 3 and 18 = inosine
Other N = A, G, C, or T
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

TGNCGHGART CDGGYTCNGA NGCRTCRAA

29

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc= "Synthetic DNA"
(ix) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(D) OTHER INFORMATION: N at 19 = inosine
Other N = A, G, C, or T
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

RTGRCTHGAR TCDGGYTCNG ANGCRTCRAA

30